Universidade de Aveiro Departamento de Biologia
2017

Catarina de Campos Telha

Performance evaluation of a novel high sensitivity troponin assay

Avaliação do desempenho de um novo ensaio de troponina de alta sensibilidade

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Avaliação do desempenho de um novo ensaio de troponina de alta sensibilidade

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Professora Doutora Maria de Lourdes Gomes Pereira, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro e do Professor Doutor David Christopher Gaze, Professor Associado do Departamento de Ciências Biomédicas da Faculdade de Tecnologia e Ciências da Universidade de Westminster (Reino Unido).

Este trabalho foi desenvolvido no Laboratório de investigação do St George's Hospital NHS Foundation Trust, London, e contou com o apoio da Singulex Inc. San Francisco, USA e do St George's Hospital NHS Foundation Trust.

o júri

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Palavras-chave Doenças cardiovasculares, biomarcadores, troponina cardiaca, ensaio de alta sensibilidade, Singulex Clarity System.

Resumo

As doenças cardiovasculares são a maior causa de morte em todo o mundo. A doença arterial coronária constitui uma das mais importantes, resultando em infarte agudo do miocárdio.

Os biomarcadores cardiovasculares desempenham um papel fundamental na avaliação dos pacientes com dor torácica que dão entrada nas urgências. As troponinas cardíacas demonstraram uma alta sensibilidade e especificidade quase absoluta para o tecido do miocárdio. São considerados os biomarcadores padrão para a detecção de lesão do miocárdio.

A introdução do ensaio de troponina de alta sensibilidade permite medir concentrações abaixo dos níveis convencionais de detecção e revelou um espectro de troponina cardíaca circulante em indivíduos saudáveis.

A fim de avaliar o desempenho analítico e clínico de um ensaio de troponina sensível, com o intuito de auxiliar no rastreio ou na detecção precoce de doenças cardiovasculares, foram realizados vários ensaios em pacientes (um estudo percentual normal, um estudo de precisão, um estudo de linearidade, um estudo de comparação de pacientes e um estudo de estabilidade).

Todas as amostras foram testadas entre o laboratório de rotina e o laboratório de Investigação no Hospital St George's NHS Foundation Trust em Londres, de acordo com os princípios éticos.

O presente estudo permitiu confirmar a sensibilidade deste novo ensaio de troponina cardíaca I e provou que o Singulex Clarity System é um aparelho com potencialidades na óptica de desempenho analítico. No entanto, devido ao seu longo tempo de manutenção, de avaliação dos controlos de qualidade e por ser um aparelho que apenas permite testar 48 amostras de cada vez, não é ainda adequado para um laboratório de rotina.

Apesar de ainda não ser utilizado no mercado, espera-se que em futuro próximo, após algumas alterações, contribua para a detecção de baixos níveis de troponina cardíaca facilitando o diagnóstico e evitando altas hospitalares inadequadas.

Keywords Cardiovascular disease, cardiovascular, cardiac troponin, high sensitivity assay, Singulex Clarity System.

Abstract

Cardiovascular disease is the largest single cause of death worldwide. The major single category of CVD is coronary artery disease resulting in acute myocardial infarction.

Cardiovascular biomarkers have played an important role in the assessment of chest pain patients in the Emergency Department. Cardiac troponins have demonstrated a high sensitivity and almost absolute myocardial tissue specificity. They are considered the standard biomarkers for the detection of myocardial injury.

Introduction of a higher sensitivity troponin assays allow measurement of concentrations below conventional levels of detection and have revealed a spectrum of circulating cardiac troponin in healthy subjects.

In order to assess the analytical and clinical performance of a sensitive troponin assay that may aid in screening for, or for early detection of CVD, a number of tests on patients (percent normal study, precision study, linearity study, patient comparison study and a stability study) were performed.

All the samples were tested between the Clinical Blood Sciences laboratory and the Research laboratory in St George's Hospital NHS Foundation Trust in London according with ethical principles.

The present study allowed to confirm the sensitivity of this new cardiac troponin I assay and proved that Singulex Clarity System is a potential analyzer in an analytical performance view. However, due to its long maintenance, quality control time evaluation and because it only allows testing 48 samples at a time, it does not have a usability required for a routine laboratory.

Although not yet used in the market, after some changes, it is expected that in the near future it will contribute to the detection of low levels of cardiac troponin facilitating the diagnosis and avoiding inappropriate hospital discharges.

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Contents

List of Figures I

List of Tables III

List of Abbreviations IV

Background 1

Chapter I – 3

Introduction 3

1.1. Cardiovascular disease 4

1.2. Cardiovascular risk factors and detection of CVD 5

1.3. Cardiovascular biomarkers 7

1.4. Cardiac troponin 10

1.4.1. Troponin complex 10

1.4.2. Cardiac troponin T 11

1.4.3. Cardiac troponin I 12

1.4.4. Troponin genetics 13

1.5. Cardiac troponin for the diagnostic of acute coronary syndrome 13

1.6. Cardiac troponin immunoassay 15

1.7. Analytical measurement of cardiac troponin 18

1.8. Cardiac troponin elevated in non-ACS conditions 20

1.9. Justification of the study 22

1.10. Aim of the Study 23

Chapter II – 24

Material and Methods 24

2.1. Organization of study 25

2.2. Local of study 25

2.3. Percent normal study 25

2.3.1. Duration and period of study....25
2.3.2. Population of study and sample size....25
2.3.3. Specimen storage....26
2.3.4. Type and sampling technique....28
2.4. Precision study....29
2.4.1. Duration and period of study....29
2.4.2. Population of study and sample size....30
2.4.3. Type and sampling technique....30
2.5. Linearity study....31
2.5.1. Duration and period of study....31
2.5.2. Population of study....31
2.5.3. Type and sampling technique....32
2.6. Patient comparison study....32
2.6.1. Duration and period of study....32
2.6.2. Population of study....32
2.6.3. Type and sampling technique....32
2.7. Stability study....33
2.7.1. Duration and period of study....33
2.7.2. Population of study....33
2.7.3. Type and sampling technique....34
2.8. Statistical treatment....34
Chapter III –....35
Results....35
3.1. Percent normal study....36
3.2. Precision study....37
3.2.1. Singulex intermediate precision data....37

3.2.2. Roche intermediate precision data....44
3.2.3. Singulex repeatability precision data....45
3.3. Linearity study....47
3.4. Patient comparison study....49
3.4.1. Abbot Architect HS cTnI Vs Singulex HS cTnI....51
3.4.2. Roche HS cTnT Vs Singulex HS cTnI....54
3.4.3. Roche HS cTnT Vs Abbot Architect HS cTnI....57
3.5. Stability study....59
Chapter IV –....65
Discussion....65
Conclusion and Future Perspectives....65
4.1 Discussion....66
4.1.1. Percent normal study....66
4.1.2. Precision study....68
4.1.3. Linearity study....69
4.1.4. Patient comparison study....69
4.1.5. Stability study....69
4.2 Conclusion and Future Perspectives....71
References....73

List of Figures

Figure 1. Global distribution of CV in males 4
Figure 2. Development of an endothelial dysfunction 5
Figure 3. Cardiac biomarkers timeline 9
Figure 4. Structure and localization of troponin complex 11
Figure 5. Cardiac troponin T 12
Figure 6. Cardiac Troponin I 12
Figure 7. Algorithm for triage of the patients presenting to the ED with chest pain suggestive of AMI 14
Figure 8. Sandwich immunoassay principal 16
Figure 9. Singulex Clarity™ System. 18
Figure 10. Comparison between the cTnI concentration and the frequency of all the samples tested. 36
Figure 11. Comparison between the age and cTnI concentration. 37
Figure 12. Comparison between the concentration (ng/L) and the CV (%) showing a precision profile for a range of 0-300 ng/L. 42
Figure 13. Comparison between the concentration (ng/L) and the CV (%) showing a precision profile for a range of 0-50ng/L. 42
Figure 14. Comparison between the concentration (ng/L) and the CV (%) showing a precision profile for a range of 0-10ng/L. 43
Figure 15. Comparison between the concentration (ng/L) and the CV (%) showing a precision profile of a range of 0-4.5ng/L. 43
Figure 16. Comparison between all the different concentration (ng/L) and the CV (%). ... 45
Figure 17. Comparison between the different dilutions with concentrations results of the diluted pools from the main pool with a concentration of 350ng/L. 48
Figure 18.Comparison between the different dilutions with concentrations results of the diluted pools from the main pool with a concentration of 25 ng/L. 49
Figure 19. Compilation between the concentrations of the same samples tested on Architect and Singulex. 51
Figure 20. Mean percentage difference between the Abbot Architect and the Singulex. 52
Figure 21. Mean absolute difference between the Abbot Architect and the Singulex. 53

Figure 22. Compilation between the concentrations of the same samples tested on Roche and Singulex. 54
Figure 23. Mean percentage difference between the Roche and the Singulex. 55
Figure 24. Mean absolute difference between the Roche and the Singulex. 56
Figure 25. Compilation between the concentrations of the same samples tested on Roche and Architect.....57
Figure 26. Mean percentage difference between the Roche and the Abbot Architect. 58
Figure 27. Mean absolute difference between the Roche and the Abbot Architect. 59
Figure 28. Variation of the concentration of pool no 1.....62
Figure 29. Variation of the concentration of pool no 2.....62
Figure 30. Variation of the concentration of pool no 3.....63
Figure 31. Variation of the concentration of pool no 4.....63

List of Tables

Table I. Reagent cartridge kit. 27
Table II. Sgx Clarity cTnI Calibrator Kit. 27
Table III. Sgx Clarity cTnI Control Kit. 27
Table IV. Sgx Clarity Buffer Kit(s) 28
Table V. Primary Sgx Clarity Consumable kit(s) 28
Table VI. Concentrations of all pools tested in the Sgx Clarity System. 38
Table VII. First twenty five results of the Singulex QC, across different days. 39
Table VIII. Compilations of the results concentrations, mean, SD, CV (%) and count for each different concentrated pool testes in the Sgx Clarity System. 411
Table IX. Mean, CV and CI of total and repeatability pools. 41
Table X. Compilations of the results concentrations, mean, SD, CV (%) and count for each different concentrated pool testes in the Cobas 8000. 44
Table XI. Mean, CV and CI of total and repeatability pools. 45
Table XII. Results of the concentration, mean, SD, count and CV (%) of the pools and the Singulex QCs tested on Sgx Clarity System 46
Table XIII. Volume of the pool with concentration of 350ng/L. 47
Table XIV. Volume of the pool with concentration of 25ng/L. 48
Table XV. Concentration results for the same patient testes in Abbot Architect cTnI assay, Sgx Clarity System cTnI assay and Roche cTnT assay. 50
Table XVI. Concentration results for the pool no 1. 60
Table XVII. Concentration results for the pool no 2. 60
Table XVIII. Concentration results for the pool no 3 60
Table XIX. Concentration results for the pool no 4. 61
Table XX. Concentration results for the pool no 5 61

List of Abbreviations

A

ACS – Acute Coronary Syndrome

Ab – Antibody

Ag – Antigen

AMI – Acute Myocardial Infarction

AST – Aspartate transaminase

C

CAD – Coronary artery disease

CI – Confidence interval

CK – Creatine kinase

CK-MB – MB isoenzyme

CRP – C-reactive protein

cTn – Cardiac Troponin

cTnI – Cardiac Troponin I

cTnT – cardiac Troponin T

CV – Coefficient of Variation

CVD – Cardiovascular disease

sTnT – sekeletal TnT

E

ECLIA – Electrochemiluminescence immunoassay

ECG – Electrocardiogram

ED – Emergency Department

EDTA – ethylenedaiminetetraacetic acid

ESC/ACC – European Society of Cardiology and the American College of Cardiology

ESRD – End stage renal disease

ETT – Exercise Tolerance Test

F

FDA – Food and Drug Administration

I

IHD – Ischemic Heart Disease

IQR – interquartile range

L

LD – Lactate Dehydrogenase

LDL – Low-density lipoprotein

S

SD – Standard deviation

SMC™ – Singulex's Single Molecule Counting

STEMI – Segment elevation myocardial infarction

STEP – Sensitive troponin evaluation project

M

MI – myocardial infarction

N

NICE – National Institute for Health and Clinical Excellence

NGI – Next generation immunodiagnostics

NSTEMI – Non-ST elevation myocardial infarction

Q

QC – Quality control

W

WHO – World Health Organization

Background

The sensitive troponin evaluation project (STEP) has been performed in the research and development laboratory at St George's Hospital NHS Foundation Trust, London. The overall supervisor of this project is Dr. Paul Collinson (paul.collinson@stgeorges.ac.uk), consultant chemical pathologist and professor of cardiovascular biomarkers in St George's Hospital NHS Foundation Trust and St George's University of London and Dr. David Gaze (D.Gaze@westminster.ac.uk), lecturer in Clinical Biochemistry at University of Westminster in London and honorary cardiac research scientist at St George's Hospital. Prof. Paul Collison is an expert advisor to the National Institute for Health and Clinical Excellence (NICE) on cardiac biomarkers, and was responsible for developing and introducing troponin and B-type natriuretics to the United Kingdom. Dr. Gaze and colleagues have won a number of awards including two distinguished Abstract wards from the National Academy of Clinical Biochemistry as well as Diploma for Oral Presentation regarding D-dimer, natriuretic peptides and cardiac troponin in dialysis patients.

The present thesis has been developed within the scope of the STEP project, to fulfill the Master of Molecular and Cellular Biology of Universidade de Aveiro.

Singulex is a commercial start-up company pioneering Next Generation Immunodiagnostics (NGI). Singulex's proprietary Single Molecule Counting (SMC™) technology is the backbone of NGI, and provides unprecedented ultra-sensitivity in the precision measurement of biomarkers. Singulex currently delivers SMC technology through its state-of-the-art clinical lab, which has performed in excess of 3 million NGI tests on more than 780000 patients. In 2016, Singulex launched the SMC-based Sgx Clarity™ instrument, a fully automated immune-analyzer, initially for NGI cardiovascular products, and to be followed by oncology, infectious disease, neurodegenerative and autoimmune diagnostic products.

Singulex agreed with a number of partners including Prof Collinson as part of a multicenter assessment of the cardiac troponin I (cTnI) assay using the Sgx Clarity™ instrument to assess the analytical performance and clinical efficacy of the novel high

sensitivity assays. Data from the evaluation will allow refinement of analyser operation and provide information to extend and independently confirm the product claims of the company. The assay is currently the CE marked and is undergoing regulatory submission to the Food and Drug Administration (FDA) in the United States.

Chapter I – Introduction

1.1. Cardiovascular disease

The term cardiovascular disease (CVD) covers a range of conditions affecting the cardiovascular system. CVD is the largest single cause of death worldwide (Figure 1). The incidence of CVD is currently higher than cancer or other non-CVD comorbid condition. It is estimated that human longevity would be extended seven years if CVD were eliminated.[1–10]

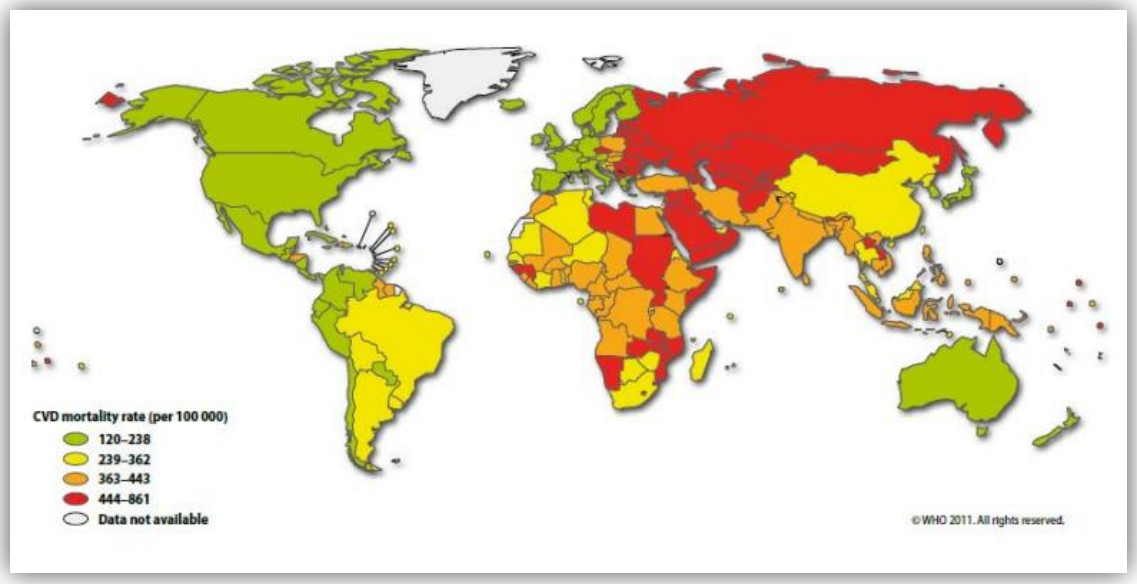

Figure 1. Global distribution of CV in males. Adapted from Mendis Puska, 2011

CVD includes ischemic heart disease (IHD), cerebrovascular disease, hypertensive heart disease, peripheral vascular disease, and structural abnormalities of the heart. The largest single category of CVD is coronary artery disease (CAD) resulting in acute myocardial infarction (AMI). [6,9,11]

CVD does not occur in single episodes but is a chronic progressive condition, beginning with plaque formation (a combination of inflammatory processes and lipid accumulation) leading to plaque destabilization, cardiac ischemia, cardiac myocyte necrosis and cardiac dysfunction.[9]

CAD is characterized by atherosclerotic plaque buildup that begins early in life and slowly progresses over time. Atherosclerosis is an inflammatory disease, with a complex pathologic process, associated with lipid and other metabolic alterations. Atherosclerosis is a systemic disorder with high prevalence and commences very early in human life.

Atherosclerosis can be affected by genetic, environmental, behavioral and dietary risk factors.[12–15]

About 50% of cardiovascular deaths occur due to sudden cardiac death. These individuals may have only one of the risk factor but a significant proportion of these sudden deaths occur without any history of CVD. [1,5,9]

Acute coronary syndrome (ACS) is a major public health problem and occurs due to the rupture or erosion of the atheromatous plaque (Figure 2). The impact of plaque disruption and vessel occlusion will depend on the vascular anatomy. The key factors are the significance of the artery to overall blood supply and the degree of collateral circulation present. A mismatch between the supply of oxygen and the demand of the tissue will occur if the affected vessel is the main source of blood flow and there is no collateral circulation. A net reduction in supply compared to the demand results in tissue ischemia. The ensuring tissue hypoxia continues causing lack of tissue perfusion and may progress to cardiomyocyte necrosis. Clinically this is known as AMI. Post AMI survival results in remodeling processes in the myocardium and the development of cardiac failure. [5,9,12]

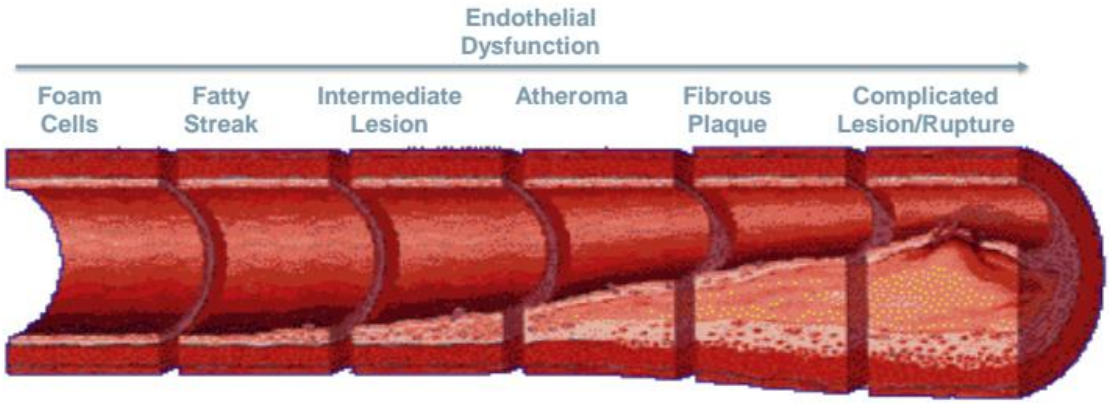

Figure 2. Development of an endothelial dysfunction, from foam cells to a ruptured atherosclerotic lesion. Adapted from Gaze, 2011.

1.2. Cardiovascular risk factors and detection of CVD

Elevated blood pressure (hypertension), usually considered as a systolic blood pressure exceeding 140 mmHg or a diastolic blood pressure exceeding 90 mmHg is one of the most prevalent risk factors for the development of CVD. [11,16] Hypertension is the most

significant single cause of cardiovascular accident (CVA, stroke) and a significant contributor to heart failure and CAD. Among the most prevalent causes of cardiovascular death are CAD, stroke, high blood pressure and heart failure.[5,14,17]

One of the other Major contributors to CVD is a diet with high sugar, sodium and fat content, together with an environmental and lifestyle changes such as reduction in physical activity.[11,12] Diet and exercise contribute to a clustering of cardiovascular risk factors including diabetes, hypertension, elevated triglycerides, increased low-density lipoprotein (LDL) cholesterol, and increased waist circumference, which are associated with progressive atherosclerotic disease.[10,18] Smoking, although on the decline in westernised countries, is a major risk factor for CVD worldwide. [11]

Morbidity and mortality from CVD is largely preventable and is one of the public health goals worldwide. There are two main strategies: preventing CVD in the at-risk population before the onset of CVD by management of traditional cardiac risk factors, or focusing on a cohort with known CVD and modifying risk factors contributing to progression of CVD. [2,11]

The first step in the clinical management and early detection of CVD is identification of high-risk individuals. This step includes preventative strategies with assessment of multiple risk factors including age, hypertension, hyperlipidemia, diabetes mellitus, positive family history, tobacco use and obesity.[2,8] Epidemiological evidence has shown that abdominal obesity is closely associated with the development of cardiovascular disease, and might be considered as an independent risk factor.[11,19] Obesity predisposes to atherosclerosis with the consequence of a shorter life expectancy.[19] It is know that ACS has a high heritability, the impact of which is far larger than the genetic effect on modifiable risk factors.[14]

The next stage is active detection of underlying CVD. Risk stratification may have direct impact on choice of subsequent tests. For CVD detection some of the commonly tools are exercise tolerance test (ETT), pharmacologic stress testing, myocardial perfusion imaging, stress echocardiography and cardiac tomography. [2] The choice of test and diagnostic sensitivity and specificity will depend on the risk category, hence prior probability of disease. Although risk assessment tools and CVD detection modalities are widely used, they are not perfect and CVD patients can be misclassified into either a low or intermediate risk group. [2,8]

The clinical symptoms of patients during the onset of ACS may include non-specific chest pain, which is one of the leading causes for visits to the hospital emergency department (ED). These visits can be caused by more than a dozen medical conditions.[3]

This complex nature of chest pain often makes it difficult for ED personnel to start necessary life-saving treatments for the patients suffering AMI. Current emergency medical evaluation of chest pain patients with suspected AMI includes obtaining the patient history, clinical examination, clinical observations (pulse, blood pressure) , the electrocardiogram (ECG), and blood testing for biomarkers of AMI, such as myoglobin, creatine kinase and cardiac troponins.[3]

The accuracy for diagnosing AMI is still relatively low, however when using signs and symptoms, ECG and cardiac specific troponin together, the diagnostic accuracy is approximately 50%.[3]

Prevention and control of CVD are possible and action is needed. In order to prevent CVD strategies are being implemented worldwide and including campaigns aimed at smoking cessation, reduction of salt consumption, increased physical activity, early detection and control of risk factors, decreasing consumption of unhealthy products, increasing physical activity and healthier diets, which is education in a proper lifestyle. [2,4,12]

1.3. Cardiovascular biomarkers

The first diagnostic test was the ECG, developed by William Einthoven. This test remains an essential tool for discriminating non-ST elevation myocardial infarction (MI) from ST elevation MI.[20]

Cardiac biomarkers, a broad subcategory of quantifiable and reproducible characteristics of biological signs, have played an important role in the diagnosis and management of patients with CVD. The largest challenge has been the identification of a biomarker with exclusive specificity for the heart, mainly because some cardiac biomarkers are not specific to a single pathway. A useful biomarker must have the following criteria: accuracy, that is the ability to identify individuals at risk; reliability, that is the stability of results when repeated at risk and therapeutic impact with early intervention.[1,9,10]

There are a number of cardiac biomarkers (Figure 3) to help in the identification of patients at risk of developing CVD, however many of this are not in use mainly because of cost, lack of an evidence base or the lack of a commercial assay. [9]

The development and introduction of new cardiac biomarkers has been increasing and evolving since the 1950s. The concept that tissue damage resulted in enzyme release that could subsequently be measured was the innovation that began the era of diagnostic enzymology. [9,20]

The first assay developed for testing for AMI was aspartate transaminase (AST). However this method was not suitable for routine clinical use and a spectrophotometric method was developed. In the meantime, it was reported that the measurement of C-reactive protein (CRP) and fibrinogen could be used for the diagnosis of AMI. CRP is released from the liver during an acute phase response and has been demonstrated to be a strong predictor of future cardiovascular events. [9,20]

The second enzyme biomarker described was lactate dehydrogenase (LD).A rise in serum LD activity was associated with AMI. However the specificity of AST and LD was the main problem. AST was raised in liver damage and LD is found in a wide range of tissues and elevated in a variety of haematological, hepatic, malignant and musculoskeletal disease states. Serum lactate measurements were used however lactate has an extremely low cardiac specificity. [9,20]

The next to appear was creatine kinase (CK), where elevated levels were associated with muscle disease, in particular muscular dystrophy. CK was considered more useful than AST, especially when there was accompanying cardiac failure. This cardiac biomarker was followed by the development of improved assays for the MB isoenzyme (CK-MB). CK-MB is the predominant isoform found in myocardial tissue and is the largest fraction of total CK, observed post MI. CK-MB supersedes AST, LD and total CK measurement by demonstrating better sensitivity and specificity but still lacks absolute cardio specificity. [9,20]

Myoglobin, a respiratory pigment found in muscle cells, was the first non-enzymatic marker used as a cardiac marker. Myoglobin also shows low cardio specificity due to elevated serum levels as a result of skeletal muscle injury or renal failure. [9]

In 1979, World Health Organisation (WHO) publicized criteria for AMI, which included the measurement of biomarkers as part of the diagnostic strategies for patients presenting with chest pain and suspected AMI. [20]

Assays for cardiac troponins (cTn) were developed for cTnI and cardiac troponin T (cTnT) in 1987 and 1989, respectively. Early studies showed that elevated troponin levels in patients with unstable angina were associated with a significant risk of cardiac events, subsequently confirmed by a large number of investigators.[20–22] In 1995 the first cTn assay, for cTnT was cleared by the FDA as an aid for the diagnosis of AMI. In 1992, the FDA cleared cTnI. [23] In 1999, the clinical chemistry community, followed by the cardiology community in 2000 recommended, cTn as the definitive biomarker for the diagnosis of AMI.[23]

When introduced cTn measurements were significantly more expensive compared to others assays. However the sensitivity and specificity for AMI diagnosis justified the cost of the new test, particularly in the ED, and the troponin measurement was "sold" as the "golden ticket". Troponin measurement was embraced with enthusiasm, and some would say clinical judgment was abandoned.[20,24]

Although the cTn assays has been established since 2000 as the preferred biomarker for the diagnostic of AMI, the original cTn assays have been progressively developed, redeveloped and reformulated to produce the current generation of sensitive and now high-sensitivity assays. [24,25]

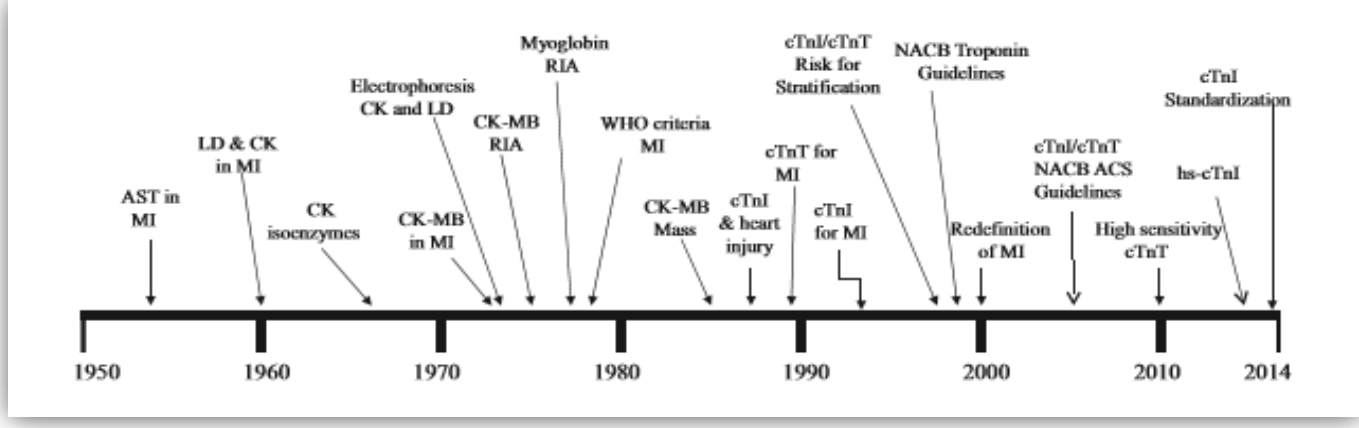

Figure 3. Cardiac biomarkers timeline, historical development of the cardiac markers. AST, aspartate transaminase; LD, lactate dehydrogenase: CK, creatine kinase, CK-MB, creatine kinase-MB isoform; WHO, World Health Organisation, cTn, cardiac troponin; cTnI, cardiac troponin, cTnT, cardiac troponin T. Adapted from Gaze & Collinson, 2005.

According to a recent update of the universal definition of AMI, published in 2007 by the Global Task Force, the diagnosis of AMI is based in the presence of unequivocal ECG changes and/or unequivocal biomarker changes; the history may be typical or atypical. Thus, the diagnosis required at least two of three criteria: positive clinical history of chest pain; unequivocal ECG changes or a rise and/or fall in cTn concentration (or CK-MB, if cTn is not available), with at least one measurement within 24 hours of the cardiac episode above the 99th percentile reference value.[20,21,23,26–33]

Biomarkers have been shown to risk stratify symptomatic and stable ACS patients for both short-term (during admission) and long-term (over 8 months to 2 years) for major adverse cardiac events. The information provided by biomarkers from various CVD pathways has enormous potential for diagnostic and prognostic applications. [1] They should be used in along with clinical findings and the results of other diagnostic modalities. Any novel cardiac biomarker should prove clinically useful information combined with cost effectiveness in order to altering patient management and reduce mortality. [9,34]

Underlying, biomarker measurement is the ability to differentiate between healthy and disease states and the documentation of disease progression.[35]

1.4. Cardiac troponin

1.4.1. Troponin complex

The troponin complex (Figure 4) (80 kDa) is located on the thin filament of the myofibril in all types of striated muscle (skeletal: slow twitch and fast twitch and cardiac muscle). It is formed from three subunits: subunit T (TnT: 37 kDa), subunit I (TnI: 22.5 kDa) and subunit C (TnC: 18 kDa) which act as a molecular switch in the presence of intracellular calcium to drive muscle contraction. The T subunit is responsible for binding to the regulatory protein tropomyosin, which is located on thin actin fibers, providing structural anchorage. TnT also modules the response to intracellular Ca^{2+} by enhancing the interaction between myosin and actin. TnI affects the myosin-actin interaction, inhibiting the actin-myosin Mg^{2+} dependent enzymatic hydrolysis of adenosine triphosphate that powers muscle contraction. TnC binds calcium ions released from the sarcoplasmic

reticulum. A number of proteins with high tissue concentrations are involved in muscle contraction however the cardiac troponins TnI and TnT are the only ones that have made the translation to a disease state biomarker due to their tissue specific isoform expression patterns. [9,10,36,37] Cardiac troponins are released from myocardial cells in case of overt myocardial injury.[10,16]

Different forms of TnT and TnI are found in cardiac and skeletal muscle, however TnC is not cardiac-specific and because of that it cannot be used in the laboratory diagnosis of cardiac injury.[36,37]

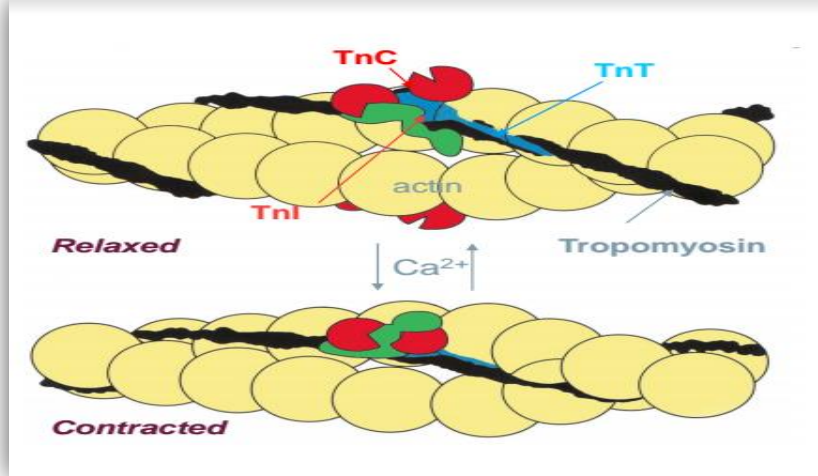

Figure 4. Structure and localization of troponin complex. Adapted from Colinson, Boa, Gaze, 2002.

1.4.2. Cardiac troponin T

cTnT meadiates the binding of troponin to tropomyosin. cTnT is a 259 residue peptide which is highly charged. Charged residues are located along the entire length of the protein making it insoluble under low physiologic ion strength. The structure (Figure 5) of N-terminal variable domain varies with regard to a ~32 amino acids portion that is not present in the skeletal isoforms of TnT. The amino terminal is rich in acidic residues and the carboxyl terminal is rich in basic residues. The molecule is 17 nm in length and rod shaped, however it has polymorphic shapes when associated with TnC and TnI. Adult cardiac muscle normally expresses a single cTnT isoform, however there are 11 isoforms produced by alternative splicing in humans.[9,36]

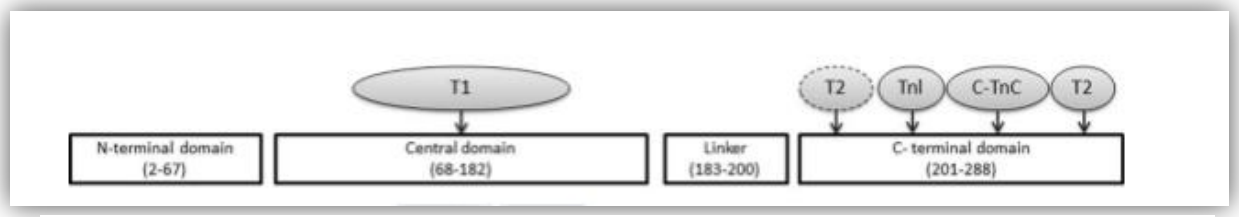

Figure 5. Cardiac troponin T – basic structure and interaction with other compounds of thin filament. Domain structure of human cardiac troponin T. Proteins of the thin filament that interact with the relevant regions of the TnT molecule are indicated in ovals. T1 and T2, regions of interaction with tropomyosin. Adapted from Katrukha , 2013.

1.4.3. Cardiac troponin I

TnI is an important modulator of myosin-actin interactions in cardiac muscle contraction and relaxation. cTnI is a 200 residue peptide and is globular in shape. It binds actin to hold the actin myosin complex in place. Conformational changes to TnI via Ca^{2+} medicated TnC dislocates TnI removing the inhibition of tropomyosin binding to myosin thus causing muscle to contract. The most significant property of cTnI is its ability to induce different conformational changes in each of the proteins that it influences. cTnI also binds tropomyosin, TnT and TnC. [9,36]

The human cTnI chain (Figure 6) consists of five domains: the N-terminal domain, the IT arm, the inhibitory domain, the regulatory domain and the C-terminal mobile domain. Residues 1-31 represent a cardiac-troponin-specific region, which plays a crucial role in the interaction of cTnI with cTnC and in the regulation of muscle contraction. [36]

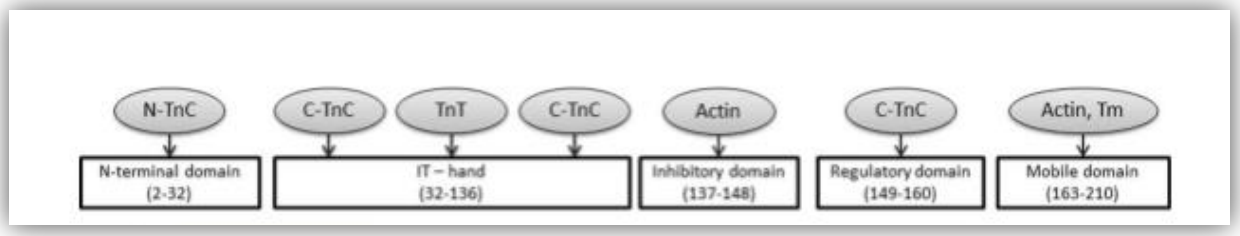

Figure 6. Cardiac Troponin I – basic structure and interaction with other compounds of thin filament. Domian structure of human cardiac troponin I. Proteins of the thin filament that interact with the relevant regions of the TnI molecule are indicated in ovals. N-TnC and CTnC, N-and C-terminal domains of TnC, respectively; Tm, tropomyosin. Adapted from Katruha, 2013

1.4.4. Troponin genetics

Skeletal and cardiac muscles develop from different embryonic cell precursors, but share a common developmental pathway. Many genes from both cell types are cross-expressed during embryonic development. There are different forms of TnI and TnT in slow twitch skeletal, fast and cardiac muscle.[9,37]

During the development of fetal muscle, all troponin isoforms are expressed simultaneously. A diversity of all three TnT could potentially exist as a result of alternative splicing of mRNA from a single gene transcript, but very few spliced variants are expressed at the protein concentration. [9,37]

Foetal cTnT isoforms are transiently expressed however these are down regulated in adult cardiac tissue. In human fetal skeletal muscle, a fetal isoform of cTnT is proportionally low in concentration. A fetal exon with sequence homology to adult fast sekeletal TnT (sTnT) and cTnT has been described. Fast or slow sTnT are not detectable in fetal cardiac tissue. Expression of embryonic isoforms in the adult heart has been associated with the deterioration of cardiac function and heart failure. [9,36,37]

At mid fetal development, the cTnT gene is up regulated in cardiac myocytes but suppressed in skeletal myocytes. cTnI is not expressed during embryonic or fetal development and is only detectable in adult cardiac tissue. During first nine months of life, sTnI is switched to cTnI expression, this means that cTnI may be not of useful value in neonatal cardiology. [9,37]

1.5. Cardiac troponin for the diagnostic of acute coronary syndrome

Cardiovascular biomarkers have played an important role in the assessment of chest pain patients in the ED. The clinical role of cTn measurement can be divided into three clinical categories (Figure 7) based on the presenting ECG and clinical assessment of the patient. These are: patients presenting with ST segment elevation myocardial infarction (STEMI); patients with questionable ACS at high risk of non-ST elevation myocardial infarction (NSTEMI) and patients with questionable ACS at low risk of NSTEMI. Patients with STEMI are highly specific for AMI and they are considered patients who will benefit from immediate revascularisation. In these patients, the role of cTn measurement is to confirm the final diagnosis. The ECG is therefore the selection test to identify patients who

will benefit from immediate treatment to salvage myocardial tissue and not as a diagnostic test for AMI. [9,24,38]

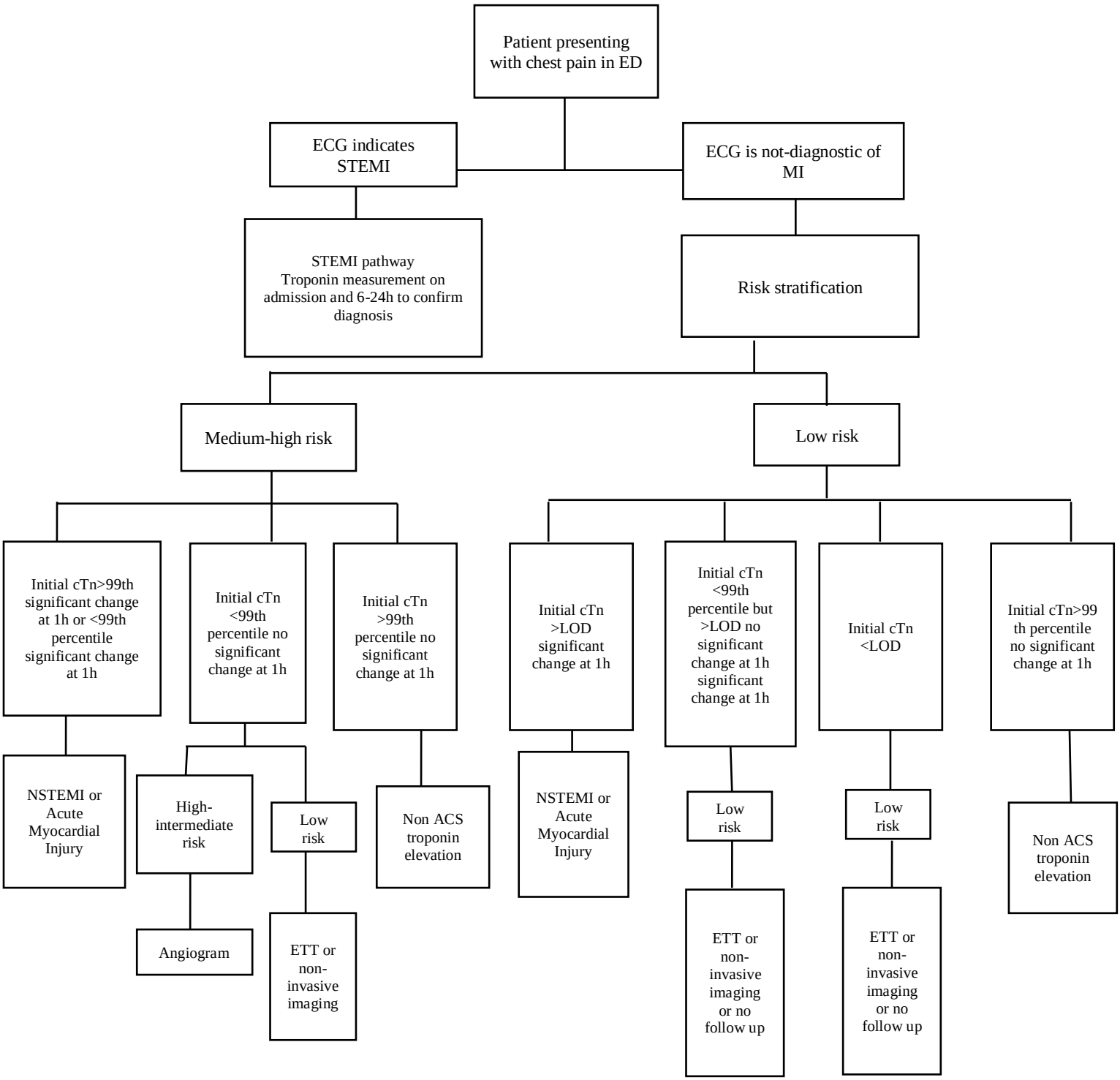

Figure 7. Algorithm for triage of the patients presenting to the ED with chest pain suggestive of AMI using high-sensitivity troponin measurements. Adapted from Paul Collinson, 2016.

1.6. Cardiac troponin immunoassay

In the last few years, several generations of assays have been available for cTnT and cTnI. The first generation of immunoassays used in the detection of cTnT was developed by Katus in 1991. Three years later kits for cTnI measurement appeared. There are many commercial cTnI assays that target different epitopes and multiple calibrators available on the market. The following high-sensitivity or ultrasensitivity cTnI are available: Abbot ARCHITECT, Beckman Acess, Singulex Erenna, Siemens Vista and Siemens ADVIA CentaurTnI-Ultra. However, the only hs cTnT assay, which is generally available, is patented under license by Roche Diagnostics GmbH (previously Boehringer-Mannheim Immunodiagnostics GmbH). [9,36]

The European Society of Cardiology and the American College of Cardiology (ESC/ACC) recommend a cutoff value at the upper 99th of values for a reference population of healthy individuals and imprecision defined as <10% for high-sensitivity cardiac troponin (hs-cTn) assay. Assays that meet this diagnostic standard are defined as contemporary sensitive. Assays that also enable cTn detection in over 50% of the general population are considered high sensitivity. This sensitivity assays allow to reduce the time interval required for diagnosing AMI but also to improve diagnostic accuracy. The majority of contemporary assays in the market currently do not meet the guidelines; however studies have provided evidence that shows that assays with an imprecision up to 20% coefficient of variation (CV) do not significantly affect diagnostic accuracy or risk stratification. The assays, which are capable of quantitating cTn at level well below the lowest cTn concentrations seen in healthy subjects should be designates as ultras-sensitive. Assays with imprecision > 20% should be used with caution.[21–24,29,32,36,39–47]

Over the past several years, manufacturers of cTn assays have improved the quality specifications of assays to allow for more precise quantitation of low cTn concentrations by introducing the highly sensitive cTn assays. The difference between these new hs assays from their predecessor is the ability to measure very low cTnT and cTnI concentrations (1-20 ng/L, well below the limit of detection of the contemporary assays) with an excellent imprecision (CV≤10%) at and below the assay's 99th percentile value. This added sensitivity allows hs cTn assays to reliably measure concentration in almost 100% of healthy individuals. Measurement of small cTn concentration changes or delta within the

normal range may provide new opportunities to screen asymptomatic populations.[23,26,28,32,34,36]

Measurement of cTnT and cTnI is by immuoassay, a biochemical test that determines the presence or the concentration of a substance in solution that frequently contains a mixture of substances. The test is based on the ability of an antibody to bind with high specificity to a specific region of the molecule being testes, the antigen. As final product of this reaction the immunoassay produce a signal in response to the antigen-antibody binding. Some immunoassays depend on the use of an analytical reagent that is associated with a detectable label. The majority of immunoassays methods for detecting cardiac biomarkers are sandwich assay (Figure 8), where the antigen (for example troponin) in the unknown is bound to specific anti-troponin capture, antibody. A secondary labelled antibody binds to form and complex antigen-antibody-antibody. The amount of labelled antibody present is measured which is directly proportional to the amount or concentration of antigen present in the sample.[9,21,36]

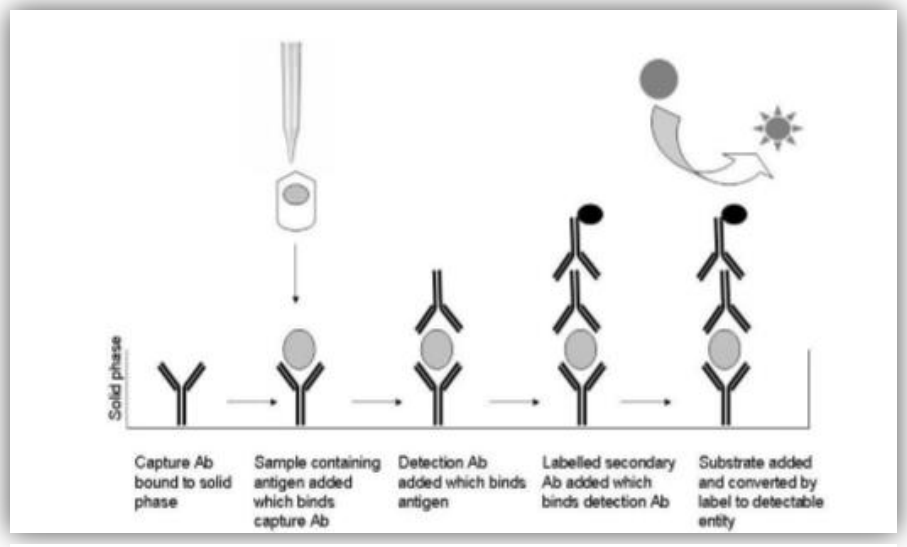

Figure 8. Sandwich immunoassay principal. Adapted from David, 2011.

The different antibody used in the commercial assays may have different sensitivities to the antigen contributing to the variations in concentrations obtained using different immunoassay systems. The electrochemiluminescence immunoassay (ECLIA) with ruthenium, the antibody of reaction, is used in the hs cTnT assay (Roche Diagnostics,

USA). Most of these assays are fully automated and are intended for use with special immunoassay analyzers.[36,37]

The recent Ultra-sensitive Erenna® Cardiac Troponin-I Immunoassay, under license by Singulex Inc. San Francisco, USA, is considered the most sensitive cTnI assay for research purposes. This hs-cTnI assay utilizes a unique immunoassay instrument with patented, digital, single-molecule detection of highly precise quantification of low-abundance biomarkers. The new assay uses single-molecule counting technology, which uses single-photon fluorescence detection. Preliminary studies demonstrated a detection limit of 0.2 ng/L and 10% CV at 1.8 ng/L, surpassed many other commercial assays. However, with such sensitive limits of detects, the possibility that low-level of nonspecific binding events contribute to cTnI measurements cannot be discarded. [36,41,43,48]

SMC™ was introduced in 2007 and since that introduction, immunoassays using the Erenna have allowed for the detection of low concentration biomarkers at levels that were previously undetectable. Several improvements have resulted in the development of an SMC™ scanning reader which forms the basis of the Singulex Clarity™ System, designed to be the most sensitive, fully-automated, next generation immunoassay platform available.[49–51]

Singulex Clarity™ System (Figure 9), a fluorescent immunoassay has been developed to measure cTnI, which utilizes SMC™. This assay utilizes two monoclonal capture antibodies and two monoclonal detection antibodies, with one of each specific for the stable central portion of cTnI, and the other specific to the N- or C- terminus to maximize its ability to detect post-translationally modified cTnI in circulation. Biotinylated capture antibody are conjugated to paramagnetic microparticles to enable capture of cTnI antigens, and fluorescently labeled detection antibodies enable quantitation by the proprietary SMC™. The capture and detection antibodies are incubated with the samples in a one-step reaction, followed by a washing step. The immunoassay sandwich is subsequently eluted from the paramagnetic microparticles with a low pH elution buffer and the fluorescent signal is measured by the Singulex Clarity System. [49,50,52]

The Erenna®, from the same company, has been shown to identify low-abundance of biomarkers with unparalleled sensitivity, precision, and accuracy. The Singulex Clarity™ System utilizes a second generation scanning-based detection system which

maintains the exquisite sensitivity of the Erenna® while improving upon the throughput, dynamic range, and usability of that system.[49,50]

There are challenges and opportunities in the high sensitivity assay era, in terms of the analytical performance and validation/interpretation procedures for the laboratory and the relevance in the clinical setting. Significant improvements have been made in performance, including standardization, precision and sensitivity. However all the troponin assays require substantial additional analytical and clinical validation before to clinical use.[20,21,27,35]

Figure 9. Singulex Clarity™ System. Adapted from A. Bartolome, 2015.

1.7. Analytical measurement of cardiac troponin

The cardiac troponins are released from the myocardium into the circulation in the first few hours following a period of ischaemia or infarction. Typically, following ACS the peak in serum concentration is seen at 12-24h.[37,40,42,53] Cardiac troponin measurement are used for the detection of myocardial damage and play a central role in aiding the diagnosis of ACS.[21,27,36,37,42,54] cTnT and cTnI have demonstrated a high sensitivity and almost absolute myocardial tissue specificity. They are the standard biomarkers for the detection of myocardial injury and prognostic evaluation of patients with ACS and without.[21,27,28,37]

At this time, a new generation of cTn assays, hs-cTn, is commercially available. These assays allow measurement of concentrations below conventional levels of detection and have revealed a spectrum of circulating cTn concentrations spanning low and high

levels in both healthy subjects and in patients with overt cardiovascular disease. Due to this clinical value, hs-cTn is been adopted as the 'gold standard' cardiac biomarker following the universal definition of AMI. cTn testing has become the standard for AMI detection as well as a challenge to clinicians to be able to discern an increased cTn between the diagnosis of AMI and a pathological mechanism in the absence of overt ischemic heart disease. [10,21–27,32,37,40–42]

Introduction of a higher sensitivity troponin assays allows diagnosis sooner than older generation assays and made possible the detection of small increases of cTn concentration. The hs-cTn assay allow and rule-in and rule-out of AMI potentially within 2-3h of admission. With these assays it may be possible to diagnose AMI earlier. Also, the use of cTnT and cTnI results in approximately 33% of patients previously classified as unstable angina being reclassified as AMI. [20–22,26,38]

Cardiac troponin measurement has been the mainstay for diagnosing ACS and offers considerable benefit for stratification of the risk for future adverse cardiac events.[21,22,48] Moreover, in individuals considered "normal" without know cardiovascular disease, increased cTn concentrations are indicative of a significantly higher risk of death.[28]

Cardiac troponins, recognized as biomarkers for AMI, are the standard biomarkers for diagnosis of myocardial damage. However, some studies in ultra-high sensitivity assays show that low levels of elevated troponin yield prognostic information for individuals without know CVD, mainly because of progressive improvement in assay sensitivity that has resulted in an increasing range of clinical conditions, where troponin elevation is detected in patients who do not have AMI. [1,24] Therefore, it has been postulated that sensitive cTn, given the detection in asymptomatic healthy people without a history of CVD; may be of value in identifying subjects at long term risk of ACS, in the other words, cTn may have a role in primary prevention.[21,33,55]

The clinical importance for a very hs-cTn assay can be notification in 3 major areas, where this assay have the potential to improve current practice: monitoring of therapeutic drugs that have the potential to cause cardiotoxicity, earlier diagnosis than is currently possible and improved stratification of the risk for future adverse cardiac events among patients being evaluated for AMI.[48]

It is important to remind that cTn elevation is not a marker of ACS itself, but a marker of cardiac cell damage acute or chronic and whatever the cause, troponin elevation is always prognostic. AMI remains a clinical diagnosis of which cTn aids in the final decision. [9,24]

1.8. Cardiac troponin elevated in non-ACS conditions

cTns are elevated in other conditions without a primary cardiac pathology, these include cardiac trauma, renal failure, autoimmune conditions, drugs and toxin induced cardiotoxicity and exercise induced release. All of those factors can contribute to confusion in the emergency clinical setting. [9]

There is a growing evidence base for the detection of cTnT and cTnI in chronic disease states, for example in patients with renal failure the troponin is commonly elevated. Studies have demonstrated that both cTnT and cTnI are elevated in renal failure and that such elevation carries prognostic significance. In patients with end stage renal disease (ESRD) cardiovascular events are the largest single cause of death. [9,37,38,56]

Another condition that contributes to an increase of troponin is a physical exercise. Practice of exercise plays an important role in the prevention of CVD. However, there is an increasing release of cTn following exercise. The mechanism by which cTn is released following prolonged or strenuous exercise remains uncertain. However, this knowledge enables physicians to make informed clinical decisions and use cTn testing appropriately in patients who present following exercise. [9,32,57]

Moreover, the release of cTn following drug therapy is also considerable interest for diagnosis, monitoring and drug safety studies. It has been documented that cocaine ingestion can induce the release of cTn and could potentially be a cause of diagnostic confusion in patients presenting with chest pain. [38]

cTn is not an exclusive biomarker for the diagnosis of AMI. It is becoming clear that multiple mechanisms contribute to troponin elevation. A number of secondary ischemic and non-ischemic cardiac injuries are known to be associated with elevated cTn, which can include necrosis, apoptosis, normal myocyte cell turnover, cellular toxicity, neurohormonal activation, release of proteolytic troponin degradation products, increased

cell-membrane permeability due to integrin-mediated “stretch” mechanisms and formation and release of membrane blebs. [21,22,36]

1.9. Justification of the study

CVD are not only debilitating for patients and their families, they also threaten the sustainability of our healthcare systems. CVD is a major contributor to rising healthcare expenditure, contributing for a large proportion of emergency care budgets. [5,21]

The condition of heart failure may be difficult to diagnose due to the presentation of non-specific symptoms. There is a risk group of patients who, if not correct diagnose, stands to be miscategorized and exposed to the potential catastrophic events of undetected CVD. Furthermore, the differential diagnosis of heart failure often requires numerous expensive procedures to obtain surrogate measures of cardiac function. [1,2,47,58]

With all the consequences of undiagnosed CVD, early detection and frequent monitoring of both traditional risk factors and novel biomarkers has the potential to save lives and reduce unnecessary costs due to CVD morbidity and mortality. [1] Clinical decision support tools are powerful process with the potential to provide faster diagnoses, modifying potential risk factors, improved prediction performance, and reduced medical costs by eliminating unnecessary testing.[1,2] However, the importance of addressing improved diagnostic capabilities during the early onset of ACS for life saving and improvement of clinical outcomes remains as an unmet global need.[3]

There is still an urgent need for identifying additional biomarkers for a more rapid detection of acute cardiac ischemia, in order to substantially improve the diagnostic accuracy, to achieve timely treatment, to improve clinical outcomes of patient survival and timeliness for initiating medical treatments. Anything which could be used to refine the initial assessment of the patient prior to referral to raise the probability of a correct diagnosis is therefore of interest.[3,22]

Furthermore, even the clinical utility to detect low levels of cTn in healthy population samples is currently unknown, cTn measurements may be very useful to improve our mechanistic understanding of cTn turnover and its associations with cardiovascular risk factors. [16]

1.10. Aim of the Study

Recently, there has been the development of hs-cTn assays for routine clinical use. Although these assays are an order of magnitude more sensitive than the original "contemporary sensitive" troponin assays, they typically have a detection limit of around 1 ng/L and do not completely measure troponin across the reference interval. The measurement of very low troponin values with low analytical imprecision may facilitate very rapid diagnosis of myocardial infarction using the recently proposed one hour rule out algorithms of the European Society of Cardiology.

The purpose of this study is to assess the analytical and clinical performance of a sensitive troponin assay that may aid in screening for, or for early detection of CVD.

The objective of the current study is the analytical validation and clinical suitability of the Singulex high sensitivity cardiac troponin I assay for routine clinical use. Then, the following tasks were planned:

- ✓ Evaluation the imprecision profile of the Singulex assay;
- ✓ Evaluation the ability of the cTnI assay on the Sgx Clarity System to detect cardiac troponin-I in self-declared healthy individuals;
- ✓ Compare the results of cardiac troponin-I from routine high-sensitivity troponin such as the Abbot STAT High Sensitive Troponin-I assay on the Abbot Architect system;
- ✓ Compare the results of cardiac troponin-I from routine high-sensitivity troponin such as the Roche Sensitive Troponin-t assay on the Cobas 8000 system;
- ✓ To assess the usability of the system for routine clinical measurement of cardiac troponin from the operator perspective and laboratory process flow impact.

Chapter II –
Material and Methods

2.1. Organization of study

This project was divided in five parts: percent normal study, precision study, linearity study, patient comparison study and stability study. All these parts of the project belong to the verification method.

Study operators were trained to the proper operation of the Sgx Clarity System and to the Instructions for Use of the Sgx Clarity cTnI assay by qualified Singulex staff before the start of study testing.

All the samples were tested in the Singulex Clarity System, in the Cobas 8000, Roche Diagnostics, and/or in the Abbot Architect.

2.2. Local of study

This project was undertaken in St George's Hospital NHS Foundation Trust in Tooting in the London Borough of Wandsworth. The samples were tested between the Clinical Blood Sciences laboratory and the Research laboratory.

2.3. Percent normal study

2.3.1. Duration and period of study

This study stared in 14 of March of 2017 and lasted until 06 of April of 2017, with duration of 4 weeks.

2.3.2. Population of study and sample size

Three hundred sixty (360) de-identified specimens from self-declared healthy donors who were not suspected to have cardiovascular disease were tested in duplicate (720 tests) using Singulex Clarity cTnI Assay. These samples were provided by the Singulex team. Informed consent was obtained from all the donors.

The selection of the individuals for the percent normal study was based on a health criterion:

- General good health;
- At least 16 years old (depending on state);

- Weigh at least 110 pounds (~49,89 Kg).

Conditions that would exclude donors:

- Hemoglobin or Hematocrit levels is below what's safe or blood donation;
- Blood pressure or pulse is too high or low;
- If the donors is under treatment for a variety of illnesses like cancer, heart disease or certain blood diseases;
- Tattoos from a non-licensed/ regulated facility;
- Body piercings;
- Cold or flu;
- Had a recent blood transfusion;
- Hepatitis;
- HIV/AIDS;
- Certain international travel (abroad diseases);
- Organ/ tissue transplants;
- Pregnancy;
- Sexually transmitted diseases.

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2.3.3. Specimen storage

Singulex provided a complete set of specimens that were shipped on dry ice, arrived frozen and were stored at -20ºC until ready to use.

Singulex also provided reagent cartridge kit (Table I), Sgx Clarity cTnI calibrator kit (Table II), Sgx Clarity cTnI control kit (Table III), Sgx clarity buffer kit(s) (Table IV) and primary Sgx Clarity consumable kit(s) (Table V). Sgx Clarity cTnI assay reagents, controls, calibrators, buffer and consumable kits were stored in accordance with the conditions described in the respective package inserts and instructions for use.

Table I. Reagent cartridge kit.

Assay Component	Description	Contents	No. of bottles	Fill volume
Reagent cartridge contents	Capture reagent: pre-diluted capture reagent (96 test capacity)	Streptavidin-labeled paramagnetic microparticles coated with Capture Antibodies, HEPES buffer, sodium azide as preservative	1	4.5 mL
	Detection reagent: pre-diluted detection reagent (96 test capacity)	Fluorescently-labeled Detection Antibodies MAbMF4 and Mab16A12, HEPES buffer, sodium azide as preservative	2	4.4 mL

Table II. Sgx Clarity cTnI Calibrator Kit.

Assay Component	Descrition	Contents	No. of Bottles	Fill Volume
cTnI calibrators	Human cardiac Troponin I calibrators	- Troponin I in human protein matrix - Target concentrations: 0 pg/mL; 17 pg/mL (range 12-35 pg/mL); 21,250 pg/mL (range 19,125-23,375 pg/mL)	3	0.75 mL

Table III. Sgx Clarity cTnI Control Kit.

Control	Target Approximate Concentration (pg/mL)
Control L-1	7.00
Control L-2	20.0
Control L-3	40.0
Control L-4	10000

Table IV. Sgx Clarity Buffer Kit(s)

Assay Component	Description	Contents	No. of Bottles	Fill Volume
Elution Buffer	General, on-board	Glycine, Triton X-100	1	30 mL
Wash Buffer	General, 10x concentrate, stored off board	Borate buffer, Triton X-100, ProClin 950 as preservative	1	1.0 L

Table V. Primary Sgx Clarity Consumable kit(s)

Consumable Component	Description	Contents	No. of Components
Detection Vessel	Clarity instrument-specific 96-well microtiter	100 detection vessels per case plus instructions for use	3 detection vessel capacity per instrument
Reaction Vessel	Clarity instrument-specific stripwells with 8 reaction wells per strip	1,600 strip wells per case plus instructions for use	8 reaction vessels per stack; 10 stacks/instrument load

2.3.4. Type and sampling technique

In this part of the study all the samples were tested in the Singulex Clarity™ cTnI assay, using single-photon fluorescence detection for the quantitation of cTnI, in ethylenedaiminetetraacetic acid (EDTA) plasma on the Sgx Clarity™ System.

The Sgx Clarity System requires a daily and weekly maintenance. Daily maintenance encompasses ‘start of the day’, where the consumables are changed and some maintenance is performed, and ‘end of the day’, where the wastes are discarded and some maintenance is performed. Weekly maintenance is performed once per week, where some

solutions are changed and some cleaning maintenance is performed. After the 'start of the day' the Sgx Clarity System is ready to start processing.

A three-point calibration of the Sgx Clarity System were performed on the first day of testing, before the first sample run on the system, and then as prompted by the Sgx Clarity System software, or when assay controls are out of specification. Three replicates of each of the 3 provided calibrators are tested in each calibration run for a total of 9 calibrator tests. A volume of 500 µL was used per each calibrator level.

After the calibration finished without any error the Sgx Clarity cTnI Controls were tested. All four levels of these controls were tested once per day, before the first run of the day. A volume of 400 µL was used per each control level. Singulex provided matching, labelled secondary tubes for loading specimen aliquots onto the Sgx Clarity System. Specimens were processed according to the instructions provided in the Sgx Clarity cTnI assay Instructions for Use. Up to 48 specimens may be included in a single run.

For each run, the aliquot tubes were removed from the freezer and they were left to thaw at room temperature for a minimum of 20 minutes. Each aliquot had 500 µL of donor plasma. The aliquots were then vortex and centrifuged at 11300 xg for 10 minutes at room temperature.

At that moment just 300 µL of each aliquot were transferred to a fresh tube, without the supernatant, before being analysed in the Sgx Clarity System.

The instrument recognized the barcodes when they were loaded and it automatically created an order.

2.4. Precision study

Samples utilised during this phase of the study were residual samples left over from routine analysis that would otherwise have been discarded and on whom testing was complete. Prior ethical approval for use of such material had been obtained.

2.4.1. Duration and period of study

This study stared in 30 of March of 2017 and lasted until 18 of April of 2017, with duration of 3 weeks.

2.4.2. Population of study and sample size

Pooled human serum samples were created using spare excess serum, no more than 48 hours old, collected from multiple anonymised patient samples, in the Clinical Blood Sciences laboratory of St George's Hospital, and pooled to form a base pool with a volume of approximately 260 mL. All patients were aged between the age of 18 and 35. All of these samples were tested on Roche equipment and all of them had a result of <3 ng/L for cTnT.

To create a pool to dilute, spare excess serum was also collected from multiple anonymized patient samples (around 20 patients) with a cardiac troponin T value > 5000 ng/L and pooled to form a concentrated pool with a volume of approximately 20 mL. All samples were collected in Chemical Pathology laboratory of St George's Hospital, and they were no more than 48 hours old.

Both pools, the base and the concentrated pool, were run on the Sgx Clarity System and the cTnI concentration of the pools was recorded.

Based on the cTnI values given by the Singulex assay, some successive dilutions were made spiked the base pool with the concentrated pool to give approximate concentrations of 0.5, 1.25, 3.0, 5.0, 10.0, 50.0, 100.0 and 300.0ng/L. These concentrations allowed to cover the range between a low limit of detection (0.5 ng/L) and a high value representative of unequivocal AMI diagnosis.

Aliquots of the different diluted pools with 400 µL each were prepared and stored at -20ºC in the freezer.

2.4.3. Type and sampling technique

In this part of the study all the samples were tested in the Singulex Clarity™ cTnI assay and on the Cobas 8000, for cTnI and cTnT, respectively. The aliquots were removed from the freezer and they were left to thaw at room temperature for a minimum of 20 minutes. The aliquots were then vortexed, centrifuged at 11300 xg for 10 minutes at room temperature, and finally the supernatant was transferred to a fresh tube before being analysed.

As part of this study an intermediate precision data and a repeatability precision data were done. For intermediate precision data studies, five aliquots of all of the different pools were measured at the same time in a single run. This procedure was performed five times either on different days or in the morning and afternoon of the same day. Theses aliquots were tested on both the Roche cTnT assay and the Singulex cTnI assay. For repeatability precision data studies, twenty aliquots of the same pool were measured at the same time on a single run. This was repeated for each of the different pools and for the different levels of the provided Singulex quality control (QC) material. This repeatability study was only performed on the Singulex cTnI assay.

In addition, to the precision data studies for the pooled samples the four different levels of the Controls provided by Singulex were also run every day the assay was used on the Singulex cTnI assay. The first twenty five results, across different days, for the QC materials were used to calculate the mean, +/- 2 standard deviation (SD) and the % of CV.

2.5. Linearity study

2.5.1. Duration and period of study

This study was performance in 18 of April of 2017.

2.5.2. Population of study

Pooled human serum samples were created using spare excess serum, no more than 48 hours old, collected from multiple anonymised patient samples, in the Chemical Pathology laboratory of St George's Hospital, and pooled to form a base pool with a volume of approximately 20 mL. All patients were aged between the age of 18 and 35.

The samples were pooled to form two pools, around 10 patients samples per each pool with volumes of approximately 10 mL per pool. The pools were tested in the Sgx Clarity System and the values for cTnI were of either approximately 350 ng/L or approximately 25ng/L

To dilute the pools (350 ng/L and 25 ng/L), pooled human serum samples were created using spare excess serum, no more than 48 hours old, collected from multiple anonymised patient samples, in the Chemical Pathology laboratory of St George's

Hospital, and pooled to form a base pool with a volume of approximately 20 mL. All of these samples were tested on Roche equipment and all of them got a result of <3 ng/L for cTnT.

2.5.3. Type and sampling technique

In this part of the study all the pools were tested in the Singulex Clarity™ cTnI assay.

The 350 ng/L and the 25 ng/L pools were diluted, in a triplicate, with the diluent pool separately in triplicate to give the following dilutions: neat, 80%, 60%, 40%, 20%, 15%, 10% and 5% in a final volume of 400µL.

The triplicate dilutions were all vortexed, centrifuged at 11300 xg for 10 minutes at room temperature, then transferred to a fresh tube before being analysed on the Singulex. Mean values of each dilution were then calculated.

2.6. Patient comparison study

2.6.1. Duration and period of study

This study was performance in 20 of April of 2017.

2.6.2. Population of study

Spare excess serum from 60 anonymised patient samples was collected from Chemical Pathology laboratory of St Helier Hospital, in London. These samples had been run on the Abbot Architect STAT HS Troponin I assay. Samples were no more than 48 hours old and had been stored at 4ºC.

2.6.3. Type and sampling technique

In this part of the study all the 60 samples were tested on the Singulex Clarity™ cTnI assay and on the Roche cTnT assay on the same day. The serum samples with a

400µL of volume were vortexed, centrifuged at 11300 xg for 10 minutes at room temperature and then transferred to a fresh tube to be analysed.

2.7. Stability study

2.7.1. Duration and period of study

This study stared in 08 of May of 2017 and lasted until 12 of May of 2017, with duration of 1 week.

2.7.2. Population of study

In order to verify the stability of Singulex cTnI assay, an experiment was performed to investigate the effects of time and temperature.

Pooled human serum samples were created using spare fresh (all samples were less than 6 hours old) excess serum collected from multiple anonymised patient samples, in the Chemical Pathology laboratory of St George's Hospital, and pooled to form a base pool with a volume of approximately 60 mL. All patients were aged between the age of 18 and 35. All of these samples were tested on Roche equipment and all of them had a result of <3 ng/L for cTnT.

To create a pool to spike the low troponin pool, spare excess serum was also collected from multiple anonymized patient samples with a high cardiac troponin T value and pooled to form a concentrated pool with a volume of approximately 20 mL. All samples were collected in Chemical Pathology laboratory of St George's Hospital.

The low troponin pool was spiked with the high troponin pool to produce different cTnI concentrations. The baseline concentration (day 0) of each of the pools was determined by running aliquots in duplicate on the Sgx cTnI Clarity System.

Aliquots of 400 µL of the different diluted pools were stored at room temperature (+/- 21ºC), -4ºC or -20ºC in the freezer.

For the 4 subsequent days (every 24 hours), two aliquots of each pool were tested on the Sgx Clarity System.

2.7.3. Type and sampling technique

In this part of the study all aliquots were tested on the Sgx Clarity System for cTnI. In each day, two frozen aliquots were removed from the freezer and they were left to thaw at room temperature for a minimum of 20 minutes. Together with two aliquots stored at room temperature and two aliquots stored at -4ºC, the aliquots were vortexed, centrifuged at 11300 xg for 10 minutes at room temperature, and transferred to a fresh tube to be analysed.

2.8. Statistical treatment

All the data was analysed using the program Analyse-IT, which was added on the Excel.

Chapter III – Results

3.1. Percent normal study

In order to evaluate the ability of the Sgx cTnI assay on the Sgx Clarity System to detect cTnI, three hundred sixty de-identified specimens from self-declared healthy donor who were not suspected to have cardiovascular disease were tested in duplicate (720 tests). From a total of 360 samples, 2 of them could not be included, one sample was missing and one was removed as outlier. All the duplicate results were averaged, resulting in a concentration range of 0.13 – 11.27 pg/mL of cTnI and a confidence interval (CI) of 90%, as shown on Figure 10.

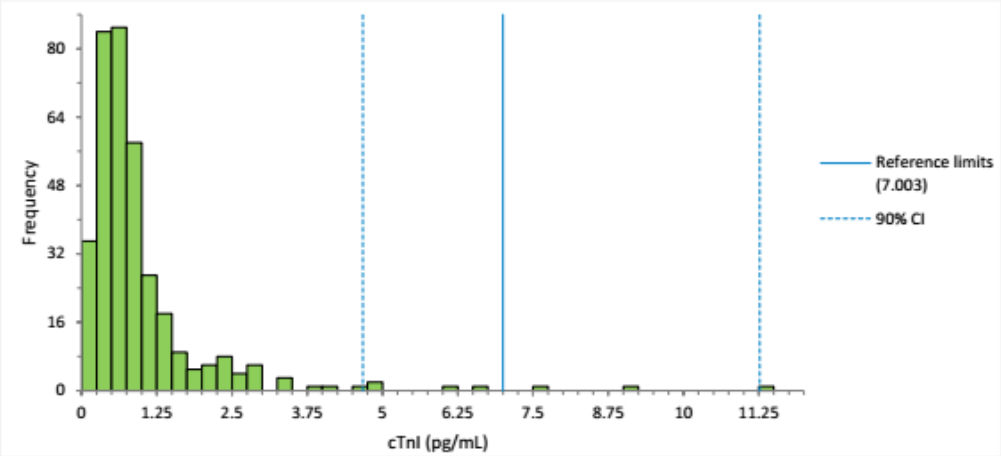
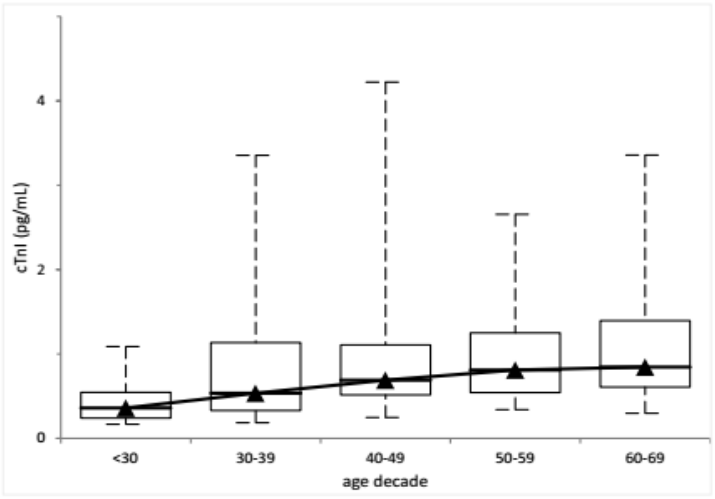
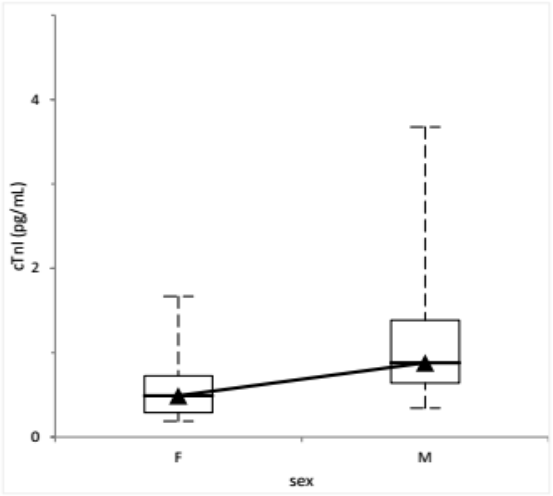


Figure 10. Comparison between the cTnI concentration and the frequency of all the samples tested.

Figure 11 compares the age and sex of each individual with the concentration of cTnI.





Mean cTnI pg/mL by Age by decade	N	1st Quartile	Median	3rd Quartile
<30	69	0.240	0.360	0.547
30-39	53	0.330	0.535	1.135
40-49	83	0.516	0.690	1.109
50-59	99	0.543	0.810	1.250
60-69	54	0.610	0.845	1.396

Mean cTnI pg/mL by Sex	N	1st Quartile	Median	3rd Quartile
F	179	0.291	0.490	0.725
M	179	0.641	0.880	1.385

Figure 11. Comparison between the age and cTnI concentration (left) and between the sex and cTnI concentration (right). The boxes represent interquartile range (IQR), mid line (triangle) represent median and whiskers represent 5th and 95th percentiles.

3.2. Precision study

3.2.1. Singulex intermediate precision data

All the pools were measured in the Sgx Clarity System. However some of them gave unexpected results (Table VI), which are being investigated by Singulex. When the result came with a “flyer” or failed sample occurred in a run, the remain samples from that pool within the run were not used to calculate precision data.

As mentioned before four different levels of QC provided by Singulex were also run every day and the first twenty five results, across different days, were used to calculate the mean, +/- 2 SDs and the % CV. All the results are shown in Table VII.

Table VI. Concentrations of all pools tested in the Sgx Clarity System.

	Pools (ng/L)							
	0.5	**1.25**	**3**	**5**	**10**	**50**	**100**	**300**
30/03/2017 (aborted rum)	0.51	1.19	2.87	4.6	9.89			
	0.45	1.21	2.93	4.23	10.2			
	0.52	1.22	2.88	4.1	10.32			
	0.52	1.14	2.97	4.33	10.22			
	0.50	0.97	3.02					
03/04/2017	0.48	222.42*	8126.55*	<0.0*	10.05	42.41	88.67	328.64
	0.52	1.03	<0.0*	4.33	9.66	42.64	89.93	327.79
	0.45	1.05	932.03*	4.24	9.48	43.60	86.48	326.02
	0.51	1.20	<0.0*	4.12	10.12	45.19	88.01	2287.22*
	0.49	217.72*	<0.0*	4.29	8.72	44.22	88.65	336.14
04/04/2017 (am)	0.40	1.15	2.75	4.11	9.41	44.35	90.28	331.09
	0.39	1.20	2.80	3.91	10.31	45.02	90.29	331.04
	0.57	1.15	2.93	6.56*	9.98	45.77	85.77	331.35
	0.52	1.26	2.88	4.15	9.57	45.48	88.60	322.33
	0.51	1.02	2.93	4.30	8.51	46.38	90.77	332.40
05/04/2017 (am)	0.53	1.26	2.51	4.22	<0.00*	43.88	91.76	327.12
	0.37	1.25	2.80	3.88	<0.00*	46.12	92.14	329.78
	0.54	1.25	2.94	4.29	<0.00*	46.37	82.06	338.29
	<0.00*	1.15	2.95	4.35	<0.00*	46.35	85.39	324.50
	0.46	1.30	2.92	4.25	9.38	47.38	87.94	323.05
05/04/2017 (pm)	0.44	<0.00*	2.83	4.58	10.05	43.12	89.67	319.18
	0.60	<0.00*	2.81	4.41	10.11	45.13	90.85	336.07
	0.62	<0.00*	2.73	4.59	10.39	44.63	87.69	328.50
	0.54	1.09	2.82	4.26	9.90	45.11	86.83	326.29
	<0.00*	1.09	3.13	4.51	9.77	51.27	89.64	334.71
06/04/2017	0.47	1.31	3.15	4.60	10.92	46.99		
	0.57	1.41	2.64	4.76	9.31	45.17		
		1.40	2.87	4.66	9.55	47.45		
		1.32	3.00		9.91	46.36		
		1.34	3.18		9.69	45.53		
11/01/2017							82.82	333.05
							89.11	338.66
							90.59	343.23
							91.42	311.71
							91.76	324.74
12/04/2017								337.96
								335.23
								330.98
								345.65
								302.75
13/04/2017 (am)	0.51	1.08		4.36	9.43			
	0.51	1.36		4.69	9.68			
	0.47	1.19		4.53	10.12			
	0.52	1.27		4.66	9.93			
	0.50	1.28		3.81	9.84			
13/04/2017 (pm)	0.49			4.31				
	0.44			4.73				
	0.52			4.47				
	0.50			4.72				
	0.51			4.09				
18/04/2017				4.66				
				4.45				
				4.72				
				4.74				
				4.78				

Mean	0.50	1.20	2.89	4.39	9.81	45.36	88.68	329.59
SD	0.05	0.11	0.15	0.26	0.48	1.87	2.64	8.81
Count	35	30	25	40	30	25	25	29
CV(%)	10.68	9.45	5.19	5.91	4.92	4.12	2.98	2.67

Table VII. First twenty five results of the Singulex QC, across different days.

Control level 1 (ng/L)			
Date	**Rep 1**	**Rep 2**	**Mean**
14/03/2017	8.68	9.36	9.02
15/03/2017	9.48	9.85	9.67
16/03/2017	8.68	8.62	8.65
17/03/2017	9.28	9.17	9.23
20/03/2017	8.97	8.84	8.91
21/03/2017	9.17	9.94	9.56
22/03/2017	9.84	10.24	10.01
28/03/2017	9.03	9.92	9.48
30/03/2017	8.76	8.95	8.86
03/04/2017	9.36	10.36	9.86
04/04/2017	9.52	9.59	9.56
05/04/2017	9.25	9.12	9.19
06/04/2017	9.74		9.74

Mean	9.35	9.36
SD	0.49	0.43
Count	25	13
CV(%)	5.26	4.54
+ 2SD	10.33	10.21
-2SD	8.36	8.51

Control level 2 (ng/L)			
Date	**Rep 1**	**Rep 2**	**Mean**
14/03/2017	26.97	28.10	27.54
15/03/2017	28.90	29.45	29.18
16/03/2017	26.91	27.65	27.28
17/03/2017	28.10	29.86	28.98
20/03/2017	27.52	27.79	27.66
21/03/2017	30.12	29.08	29.60
22/03/2017	29.02	28.76	28.89
28/03/2017	27.49	29.08	28.29
30/03/2017	27.22	27.22	27.22
03/04/2017	26.55	28.11	27.33
04/04/2017	28.34	28.45	28.40
05/04/2017	27.73	28.19	27.96
06/04/2017	29.69		29.69

Mean	28.25	28.31
SD	0.97	0.89
Count	25	13
CV(%)	3.45	3.14
+ 2SD	30.20	30.08
-2SD	26.30	26.53

Control level 3 (ng/L)			
Date	**Rep 1**	**Rep 2**	**Mean**
14/03/2017	65.58	65.77	65.68
15/03/2017	68.02	67.91	67.97
16/03/2017	64.07	62.97	63.52
17/03/2017	67.18	66.51	66.85
20/03/2017	69.87	65.94	67.91
21/03/2017	69.37	68.86	69.12
22/03/2017	68.25	68.47	68.36
28/03/2017	68.14	70.34	69.24
30/03/2017	64.71	64.11	64.41
03/04/2017	68.27	69.46	68.87
04/04/2017	67.45	68.36	67.91
05/04/2017	68.27	73.03	70.65
06/04/2017	71.20		71.20

Mean	67.68	67.82
SD	2.35	2.24
Count	25	13
CV(%)	3.47	3.30
+ 2SD	72.38	72.30
-2SD	32.99	63.34

Control level 4 (ng/L)			
Date	**Rep 1**	**Rep 2**	**Mean**
14/03/2017	14518.13	14614.15	14566.14
15/03/2017	14444.44	14292.14	14368.29
16/03/2017	14395.82	14371.37	14383.60
17/03/2017	14439.33	14273.05	14356.19
20/03/2017	15576.97	15173.69	15375.33
21/03/2017	14767.55	14804.32	14785.94
22/03/2017	14513.00	14568.00	14540.50
28/03/2017	14762.00	15432.00	15097.00
30/03/2017	15035.80	14771.71	14903.76
03/04/2017	15336.81	15515.73	15426.27
04/04/2017	15766.22	15856.64	15811.43
05/04/2017	15530.02	14931.20	15230.61
06/04/2017	15024.88		15024.88

Mean	489.36	14913.07
SD	489.36	464.93
Count	25	13
CV(%)	3.28	3.12
+ 2SD	15887.32	15842.93
-2SD	13929.87	13983.21

Only samples highlighted in green were used for precision calculations, even if they were not tested in the same day, together with pools with different concentration.

The compilations of the results for each different concentrated pool are shown in Table VIII. These results include all the different pools, the mean for each pool, SD and CV in a count of 25 runs per each pool, in one run per day with five replicates per run.

The results for CV and CI are shown on Table IX.

Table VIII. Compilations of the results concentrations, mean, SD, CV (%) and count for each different concentrated pool testes in the Sgx Clarity System.

Pools (ng/L)							
0.5	**1.25**	**3**	**5**	**10**	**50**	**100**	**300**
0.51	1.19	2.87	4.22	10.05	42.41	88.67	331.09
0.45	1.21	2.93	3.88	9.66	42.64	89.93	331.04
0.52	1.22	2.88	4.29	9.48	43.60	86.48	331.35
0.52	1.14	2.97	4.35	10.12	45.19	88.01	322.33
0.50	0.97	3.02	4.25	8.72	44.22	88.65	332.40
0.48	1.15	2.75	4.58	9.41	44.35	90.28	327.12
0.52	1.20	2.80	4.41	10.31	45.02	90.29	329.78
0.45	1.15	2.93	4.59	9.98	45.77	85.77	338.29
0.51	1.26	2.88	4.26	9.57	45.48	88.60	324.50
0.49	1.02	2.93	4.51	8.51	46.38	90.77	323.05
0.40	1.26	2.51	4.36	10.05	43.88	91.76	319.08
0.39	1.25	2.80	4.69	10.11	46.12	92.14	336.07
0.57	1.25	2.94	4.53	10.39	46.37	82.06	328.50
0.52	1.15	2.95	4.66	9.90	46.35	85.39	326.29
0.51	1.30	2.92	3.81	9.77	47.38	87.94	334.71
0.51	1.31	2.83	4.31	10.92	43.12	89.67	33.05
0.51	1.41	2.81	4.73	9.31	45.13	90.85	338.66
0.47	1.40	2.73	4.47	9.55	44.63	87.69	343.23
0.52	1.32	2.82	4.72	9.91	45.11	86.83	311.71
0.50	1.34	3.13	4.09	9.69	51.27	89.64	342.74
0.49	1.08	3.15	4.66	9.43	46.99	82.82	337.96
0.44	1.36	2.64	4.45	9.68	45.17	89.11	335.23
0.52	1.19	2.87	4.72	10.12	47.45	90.59	330.98
0.50	1.27	3.00	4.74	9.93	46.36	91.42	345.65
0.51	1.28	3.18	4.78	9.84	46.36	91.76	302.75

Mean	0.49	1.23	2.89	4.44	9.78	45.36	88.68	329.59
SD	0.04	0.11	0.15	0.26	0.50	1.87	2.64	2.85
CV	8.15	8.84	5.19	5.93	5.10	4.12	2.98	2.85
Count	25	25	25	25	25	25	25	25

Table IX. Mean, CV and CI of total and repeatability pools.

Runs per day	1
Replicates per run	5

			Total		Repeatability	
Coded Concentration	Days	Mean	CV	95% CI	CV	95% CI
0.49	5	0.492	8.7%	6.7% to 12.3%	8.7%	6.7% to 12.6%
1.23	5	1.227	9.2%	6.5% to 15.6%	6.8%	5.2% to 9.8%
2.89	5	2.890	5.3%	4.1% to 7.4%	5.3%	4.1% to 7.7%
4.44	5	4.442	6.1%	4.6% to 9.0%	5.3%	4.0% to 7.6%
9.78	5	9.776	5.2%	4.1% to 7.2%	5.2%	4.0% to 7.5%
45.36	5	45.357	4.2%	3.2% to 5.9%	3.9%	3.0% to 5.7%
88.68	5	88.685	3.2%	2.5% to 4.5%	3.2%	2.4% to 4.6%
329.59	5	329.586	3.1%	2.4% to 4.5%	3.1%	2.4% to 4.5%

With all the results and focused on Singulex cTnI intermediate precision some precision profile figures were made. They compare the concentration (ng/L) with the CV (%) (Figures 12-15).

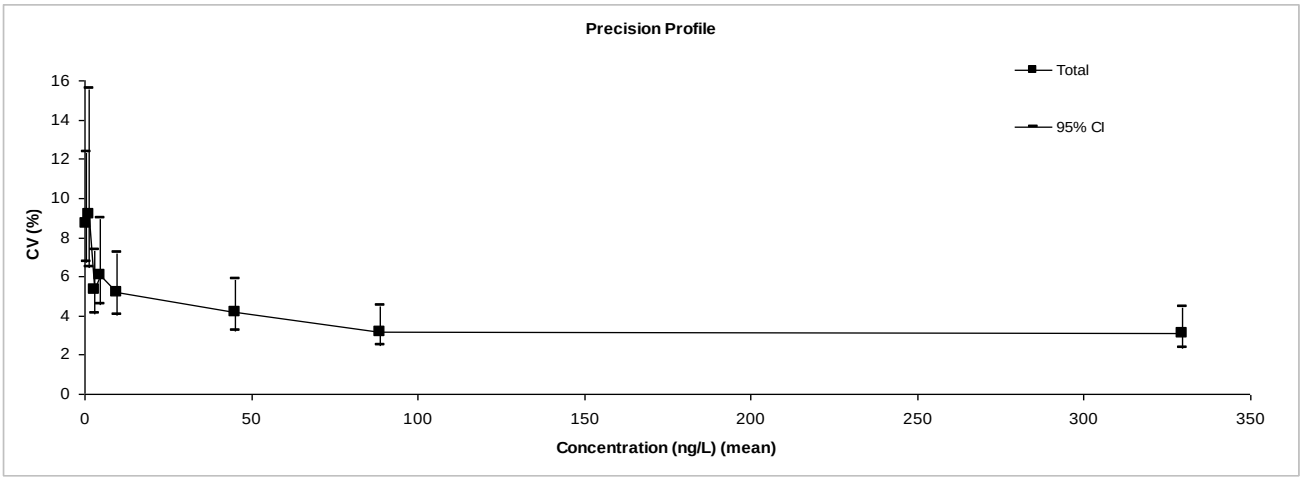


Figure 12. Comparison between the concentration (ng/L) and the CV (%) showing a precision profile for a range of 0-300 ng/L.

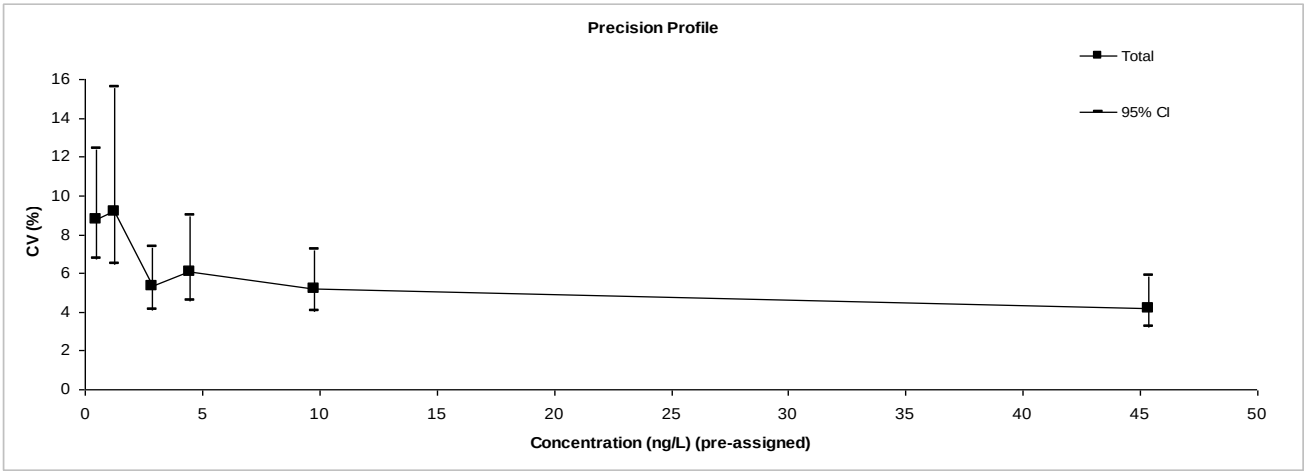


Figure 13. Comparison between the concentration (ng/L) and the CV (%) showing a precision profile for a range of 0-50ng/L.

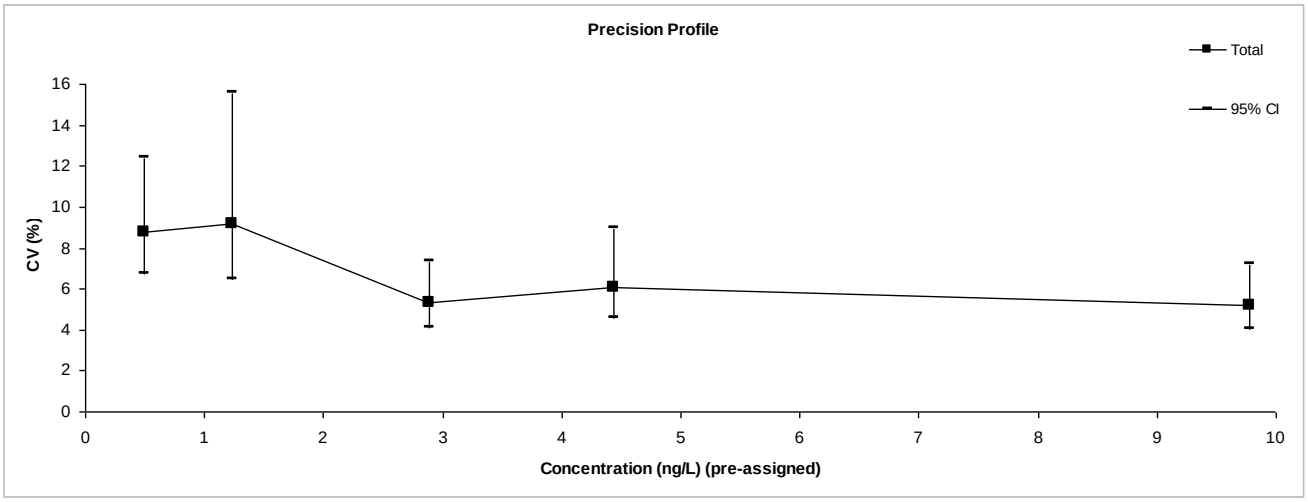


Figure 14. Comparison between the concentration (ng/L) and the CV (%) showing a precision profile for a range of 0-10ng/L.

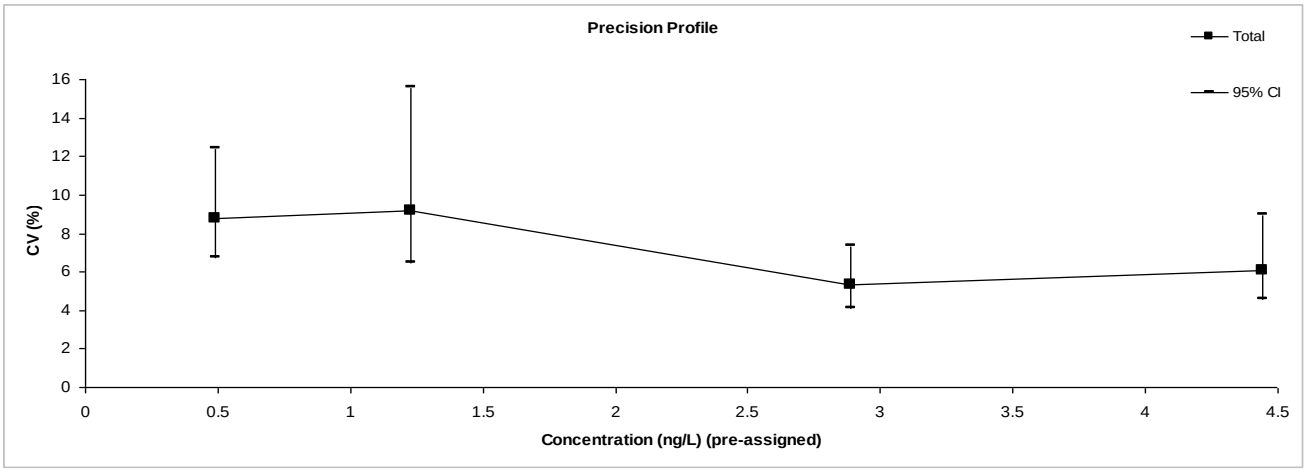


Figure 15. Comparison between the concentration (ng/L) and the CV (%) showing a precision profile of a range of 0-4.5ng/L.

3.2.2. Roche intermediate precision data

All the pools tested on Sgx Clarity System were also tested on Cobas 8000 cTnT assay. The compilations of the results for each different concentrated pool are shown in Table X. These results include all the different pools, the mean for each pool, SD and CV in a count of 25 runs per each pool, in one run per day with five replicates per run. CV and CI were also calculated and are shown on Table XI.

Table X. Compilations of the results concentrations, mean, SD, CV (%) and count for each different concentrated pool testes in the Cobas 8000.

		Pools (ng/L)				
	Repeat	5	10	50	100	300
03/04/2017	1	5.70	43.87	39.07	65.32	169.20
	2	5.74	44.21	38.65	65.69	171.20
	3	5.63	44.44	38.91	66.17	171.30
	4	5.40	43.88	38.63	66.28	168.60
	5	5.64	43.64	39.32	65.75	170.90
04/04/2017 (am)	6	5.50	45.14	39.04	65.89	168.70
	7	5.24	44.93	38.96	66.73	169.40
	8	5.43	44.37	39.58	65.85	170.90
	9	5.78	44.20	39.36	66.62	166.90
	10	5.31	42.95	39.46	66.82	170.10
04/04/2017 (pm)	11	5.24	44.03	38.83	66.21	168.50
	12	5.54	43.55	39.77	65.90	169.60
	13	5.39	44.47	39.57	66.58	169.80
	14	5.67	44.20	38.06	65.84	173.00
	15	5.94	44.51	38.65	66.85	169.10
05/04/2017 (am)	16	5.73	45.85	41.42	69.26	178.30
	17	5.18	46.05	40.31	68.73	177.80
	18	4.84	46.46	39.91	68.95	177.40
	19	5.45	46.47	39.95	68.95	180.50
	20	5.70	45.94	40.00	69.95	177.10
05/04/2017 (pm)	21	5.14	46.33	40.99	69.63	179.50
	22	5.47	45.49	41.21	70.41	179.50
	23	5.07	46.02	40.98	69.01	179.10
	24	4.98	45.56	41.22	69.66	178.80
	25	5.15	46.64	40.64	69.86	179.60
Mean		5.43	44.93	39.70	67.48	173.39
SD		0.28	1.07	0.95	1.71	4.66
Count		25	25	25	25	25
CV (%)		5.14	2.39	2.38	2.53	2.69

Table XI. Mean, CV and CI of total and repeatability pools.

n	25					
Runs per day	1					
Replicates per run	5					
			Total		Repeatability	
Coded Concentration	Days	Mean	CV	95% CI	CV	95% CI
5.4344	5	5.434	5.2%	4.0% to 7.8%	4.6%	3.5% to 6.6%
39.6996	5	39.700	2.6%	1.6% to 5.6%	1.2%	0.9% to 1.7%
44.928	5	44.928	2.6%	1.7% to 5.7%	1.1%	0.9% to 1.6%
67.4764	5	67.476	2.8%	1.7% to 7.9%	0.7%	0.5% to 1.0%
173.392	5	173.392	2.9%	1.8% to 8.4%	0.8%	0.6% to 1.1%

With all the results and focused on Roche cTnT intermediate precision some profile figure was made. In the Figure 16 all the different concentration (ng/L), representative of mean, are compared with the CV (%).

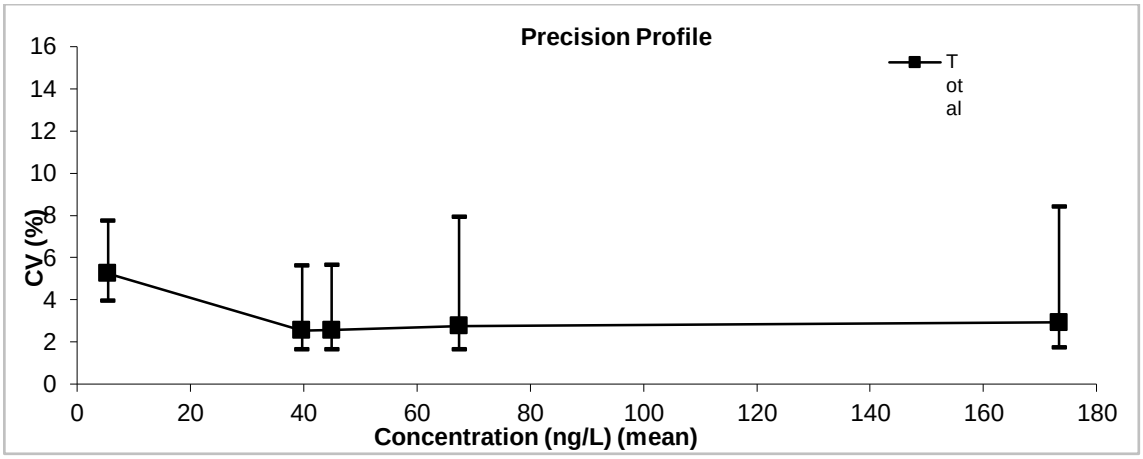


Figure 16. Comparison between all the different concentration (ng/L) and the CV (%).

3.2.3. Singulex repeatability precision data

For repeatability precision data studies, twenty aliquots of the same pool were measured at the same time on a single run within two different days. This study was only performed on the Sgx Clarity System.

The results for the different pools and Singulex QCs are shown on Table XII in an increscent order from the lowest to the higher concentration. The mean, SD, count and CV are also showing in the same table as well as the days were the samples were tested.

Table XII. Results of the concentration, mean, SD, count and CV (%) of the pools and the Singulex QCs tested on Sgx Clarity System. The results are show in an increased concentration order.

Sample	0.5 pool (ng/L)	1.25 pool (ng/L)	3.0 pool (ng/L)	5.0 pool (ng/L)	Singulex 1 (ng/L)	10.0 pool (ng/L)
1	0.51	0.98	2.45	4.36	9.35	9.43
2	0.51	1.03	2.60	4.69	9.49	9.68
3	0.47	1.16	2.72	4.53	9.38	10.12
4	0.52	1.04	2.76	4.66	9.61	9.93
5	0.50	1.20	2.88	3.81	10.14	9.84
6	0.50	1.24	2.91	4.23	10.51	9.95
7	0.52	1.20	2.70	4.12	10.50	10.13
8	0.54	1.12	2.92	4.40	10.52	10.38
9	0.50	1.03	2.40	4.65	10.24	8.89
10	0.51	1.08	2.59	4.44	10.39	9.67
11	0.47	1.11	3.00	4.45	10.69	10.16
12	0.50	1.16	2.91	4.61	10.83	9.28
13	0.48	1.09	2.63	3.96	10.00	9.36
14	0.49	1.24	2.83	4.33	9.98	10.30
15	0.51	1.23	2.79	4.34	10.43	10.47
16	0.48	1.18	3.06	4.50	9.01	10.28
17	0.56	1.24	2.83	4.46	9.16	10.59
18	0.47	1.22	3.00	4.25	9.28	10.09
19	0.52	1.31	2.17	4.44	9.01	11.70
20	0.52	1.06	2.75	4.66	9.60	9.12
Date	**13/04/2017**	**13/04/2017**	**13/04/2017**	**13/04/2017**	**13/04/2017**	**13/04/2017**
Mean	0.50	1.15	2.75	4.40	9.91	9.97
SD	0.02	0.09	0.22	0.23	0.60	0.62
Count	20	20	20	20	20	20
CV (%)	4.66	7.90	8.13	5.31	6.02	6.23

Sample	Singulex 2 (ng/L)	50.0 pool (ng/L)	Singulex 3 (ng/L)	100 pool (ng/L)	300 pool (ng/L)	Singulex 4 (ng/L)
1	26.35	47.53	66.13	88.34	337.96	14097.06
2	27.70	47.55	67.16	87.18	335.23	16054.25
3	27.97	47.29	67.75	91.39	330.98	16191.86
4	28.71	46.57	71.09	91.05	345.65	15781.40
5	28.42	45.21	63.38	91.05	302.75	16604.41
6	29.11	45.08	62.65	92.05	317.29	15819.05
7	28.69	48.15	66.98	89.37	322.42	15709.00
8	29.93	49.10	67.10	94.23	334.07	16111.17
9	27.50	46.97	67.92	79.66	332.81	14644.14
10	26.39	49.37	68.79	95.91	324.81	15201.57
11	27.61	47.87	66.52	93.50	335.63	15480.57
12	27.73	48.95	68.87	88.49	331.65	16111.09
13	28.92	44.70	58.44	90.58	322.37	15587.98

14	29.19	47.06	59.03	90.61	321.04	15292.67
15	28.30	47.26	62.71	92.44	328.99	15862.73
16	28.38	47.81	66.33	94.11	324.80	15464.75
17	25.58	48.41	65.23	94.32	343.47	15543.54
18	26.71	48.32	64.57	94.20	326.41	15570.35
19	28.15	48.19	68.34	92.44	365.00	15386.91
20	27.81	47.49	65.38	85.23	334.55	14347.66
Date	**12/04/2017**	**12/04/2017**	**12/04/2017**	**12/04/2017**	**12/04/2017**	**12/04/2017**
Mean	27.96	47.44	65.72	90.31	330.89	15543.11
SD	1.08	1.28	3.20	3.71	12.51	618.20
Count	20	20	20	20	20	20
CV (%)	3.85	2.70	4.86	4.11	3.78	3.98

3.3. Linearity study

As part of linearity study two pools with a concentration of 350 ng/L and 25ng/L were diluted with a base pool separately in triplicate to give the following dilutions: neat, 80%, 60%, 40%, 20%, 15%, 10% and 5% in a final volume of 400µL. The triplicate dilutions were measured on Sgx System Clarity and the results for the concentration of the successive dilutions are show on Table XIII and XIV, for the 350ng/L pool and 25ng/L pool, respectively. The same tables include the volume of the pool concentrated and the volume of the base pool used to get the % required.

Table XIII. Volume of the pool concentrated and of the base pool used, as well as the concentration of the diluted pools from the main pool with a concentration of 350ng/L.

Sample	350 pool (µL)	Diluent (µL)	Concentration
100%	400	0	350
80%	320	80	280
60%	240	160	210
40%	160	240	140
20%	80	320	70
15%	60	340	52.5
10%	40	360	35
5%	20	380	17.5

Table XIV. Volume of the pool concentrated and of the base pool used, as well as the concentration of the diluted pools from the main pool with a concentration of 25ng/L.

Sample	27 pool (µL)	Diluent (µL)	Concentration
100%	400	0	27
80%	320	80	21.6
60%	240	160	16.2
40%	160	240	10.8
20%	80	320	5.4
15%	60	340	4.05
10%	40	360	2.7
5%	20	380	1.35

With all the results and focused on Singulex cTnI linearity study some profile figures were made. In this figures the different dilution (%) were compared with the concentration results (ng/L). The results shown on Figure 17 and Figure 18 are related to pool with a concentration of 350ng/L and 25ng/L, respectively.

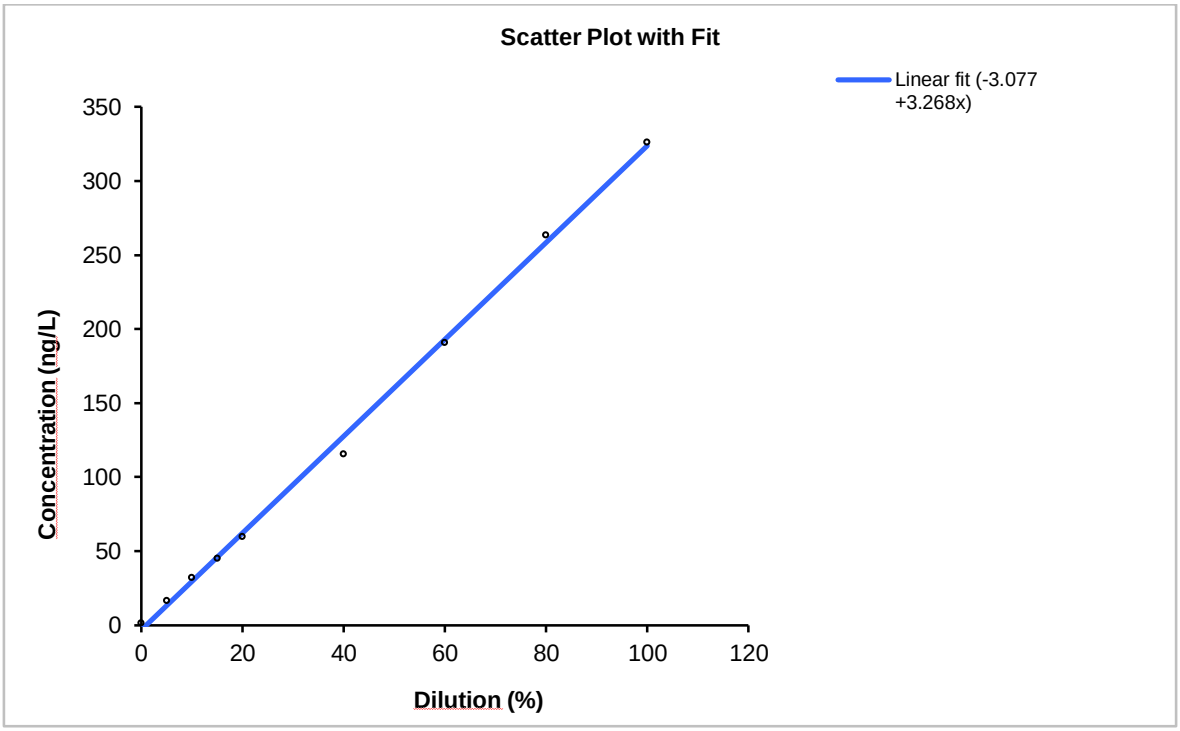


Figure 17. Comparison between the different dilutions with concentrations results of the diluted pools from the main pool with a concentration of 350ng/L.

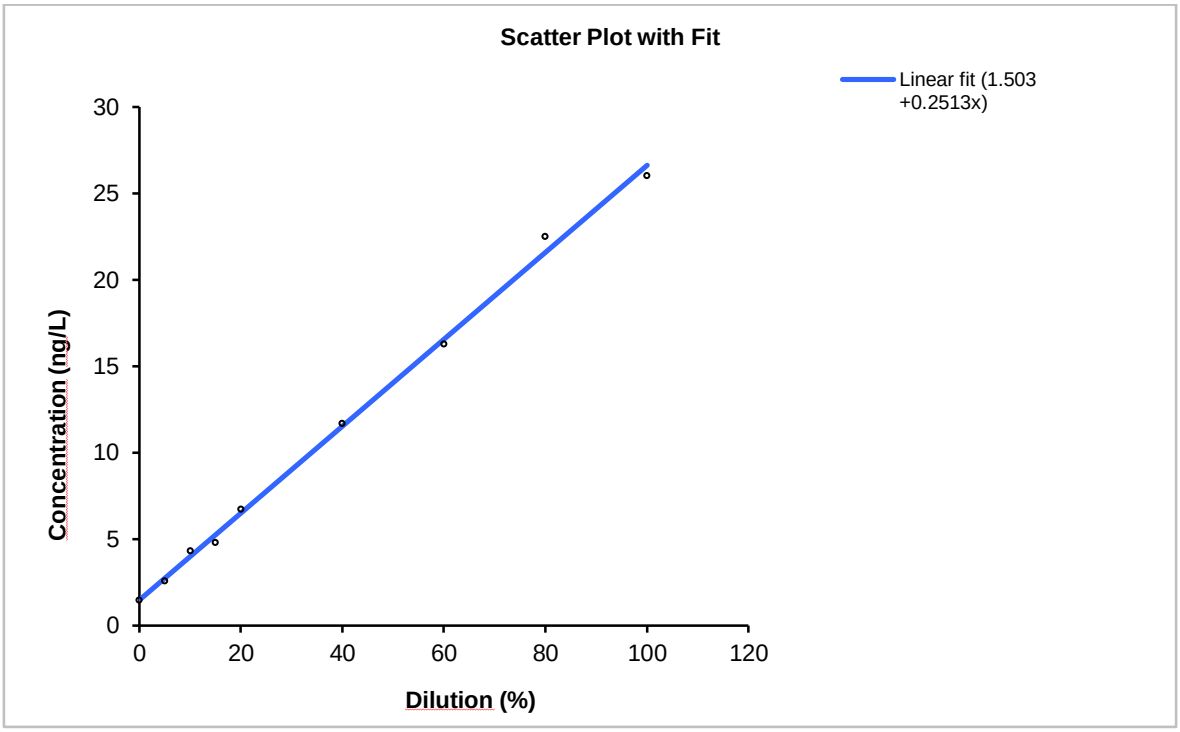


Figure 18.Comparison between the different dilutions with concentrations results of the diluted pools from the main pool with a concentration of 25 ng/L.

3.4. Patient comparison study

In order to compare the results from Singulex cTnI assay with a cTnI assay from a different company, and with a cTnT assay, some spare excess serum was collected from sixty anonymised patients' samples that had been run on the Abbot Architect STAT HS Troponin I assay.

In the same day all sixty samples were processed on Sgx Clarity System and Cobas 8000 from Roche. The concentrations results for all 60 samples tested on the three different analysers (Abbot Architect, Sgx Clarity System and Cobas 8000) are shown on Table XV.

Table XV. Concentration results for the same patient testes in Abbot Architect cTnI assay, Sgx Clarity System cTnI assay and Roche cTnT assay.

Sample number	Architect cTnI (ng/L)	Singulex cTnI (ng/L)	Roche cTnT (ng/L)
1	9	5.59	7.96
2	20	9.51	54.37
3	71	57.54	101.00
4	230	91.82	11160
5	72	5.6	19.83
6	28	10.19	30.19
7	<2*	1.52	<3*
8	4	1.72	17.84
9	100	19.74	67.53
10	76	28.32	168.80
11	13	9.26	14.66
12	4	2.55	25.47
13	2	1.89	<3*
14	95	52.64	56.91
15	30	13.02	35.23
16	<2*	0.35	<3*
17	112	12.59	10.32
18	5	2.51	17.98
19	30	24.38	43.59
20	17	6.68	26.41
21	9	4.47	5.54
22	88	117.23	26.11
23	<2*	1.44	6.96
24	28	15.64	42.65
25	7	3.59	5.12
26	24	11.91	29.86
27	67	49.58	58.24
28	50	9.29	20.91
29	10	4.59	13.73
30	4	3.28	4.300
31	106	110.82	32.32
32	85	60.37	51.02
33	52	8.51	21.53
34	4	2.99	4.01
35	4	2.86	4.09
36	3	2.30	<3*
37	3	2.06	12.45
38	3	1.37	<3*
39	57	30.02	23.47
40	<2*	0.93	<3*
41	50	23.22	60.95
42	27	14.11	28.94
43	14	8.96	8.94
44	10	7.20	7.92
45	2	1.54	<3*
46	25	17.52	16.44
47	<2*	1.17	<3*
48	3	2.30	11.18
49	5	3.08	10.06
50	9	6.49	28.40
51	<2*	0.78	<3*
52	6	3.88	7.01
53	2	2.69	9.29
54	<2*	0.92	<3*
55	6	4.00	7.17
56	<2*	1.77	4.54
57	10	7.56	36.41
58	7	11.81	35.76
59	50	16.62	58.32
60	<2*	0.92	<3*

* Samples excluded from the calculations.

3.4.1. Abbot Architect HS cTnI Vs Singulex HS cTnI

From all 60 samples tested a total of 51 samples were used in the analysis, 9 samples could not be included once they did not have an absolute value i.e. <2 ng/L. Samples ranged from 2 to 230ng/L for the Architect cTnI method were used to the analysis. Figure 19 shows the compilation between the same samples tested on Architect and Singulex.

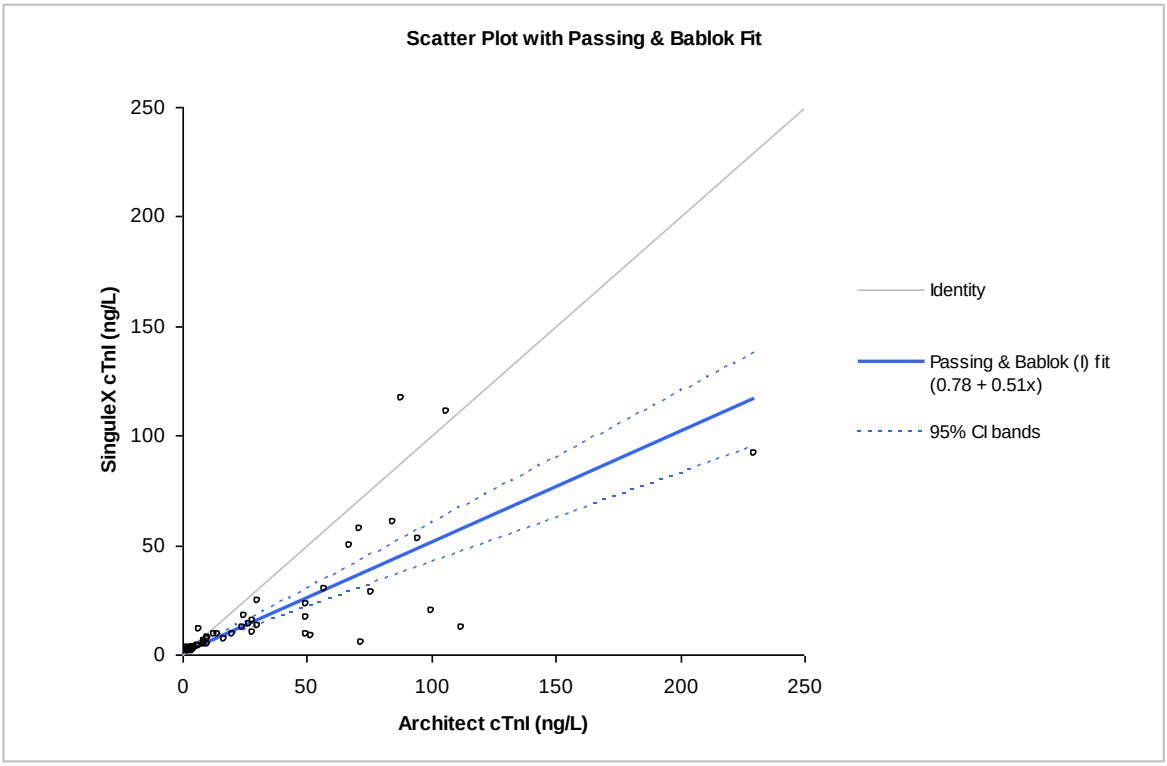


Figure 19. Compilation between the concentrations of the same samples tested on Architect and Singulex.

In order to compare both analysers and found the bias between them a figure was constructed. Figure 20 shows the relation between the differences from Singulex concentration to Abbot Architect concentration dividing by the mean of all the samples related to the mean of all the samples tested. Therefore, the figure shows the mean percentage of difference between the Singulex and Abbot with a result of – 54.5%.

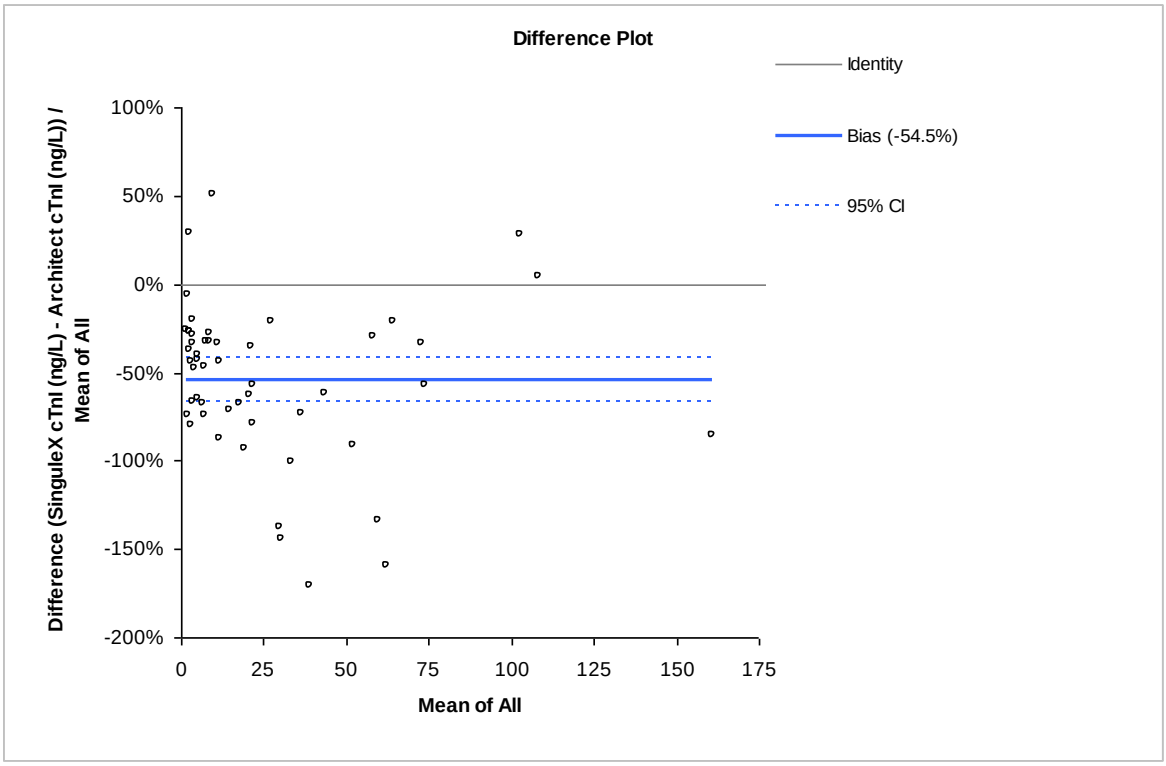


Figure 20. Mean percentage difference between the Abbot Architect and the Singulex.

Moreover, the mean absolute of the difference between the Singulex and Abbot was calculated and shown on Figure 21. The figure shows the relation between the differences from Singulex concentration to Abbot Architect concentration related to the mean of all the samples tested. In this figure the result for the mean was -16.1 ng/L.

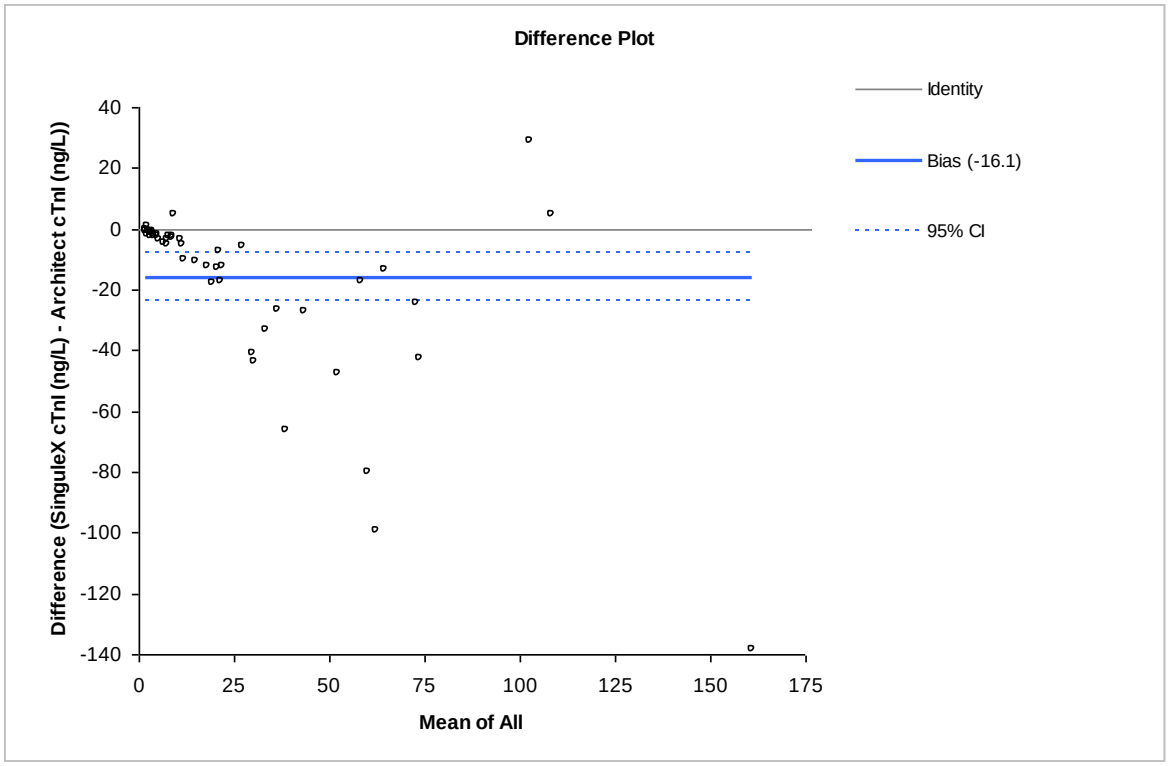


Figure 21. Mean absolute difference between the Abbot Architect and the Singulex.

3.4.2. Roche HS cTnT Vs Singulex HS cTnI

From all 60 samples tested a total of 49 samples were used in the analysis, 11 samples could not be included since they did not have an absolute value i.e. <3.00 ng/L. Samples ranged from 41.0 to 168.8ng/L for the Roche cTnT method were used to the analysis. Figure 22 shows the compilation between the same samples tested on Roche and Singulex.

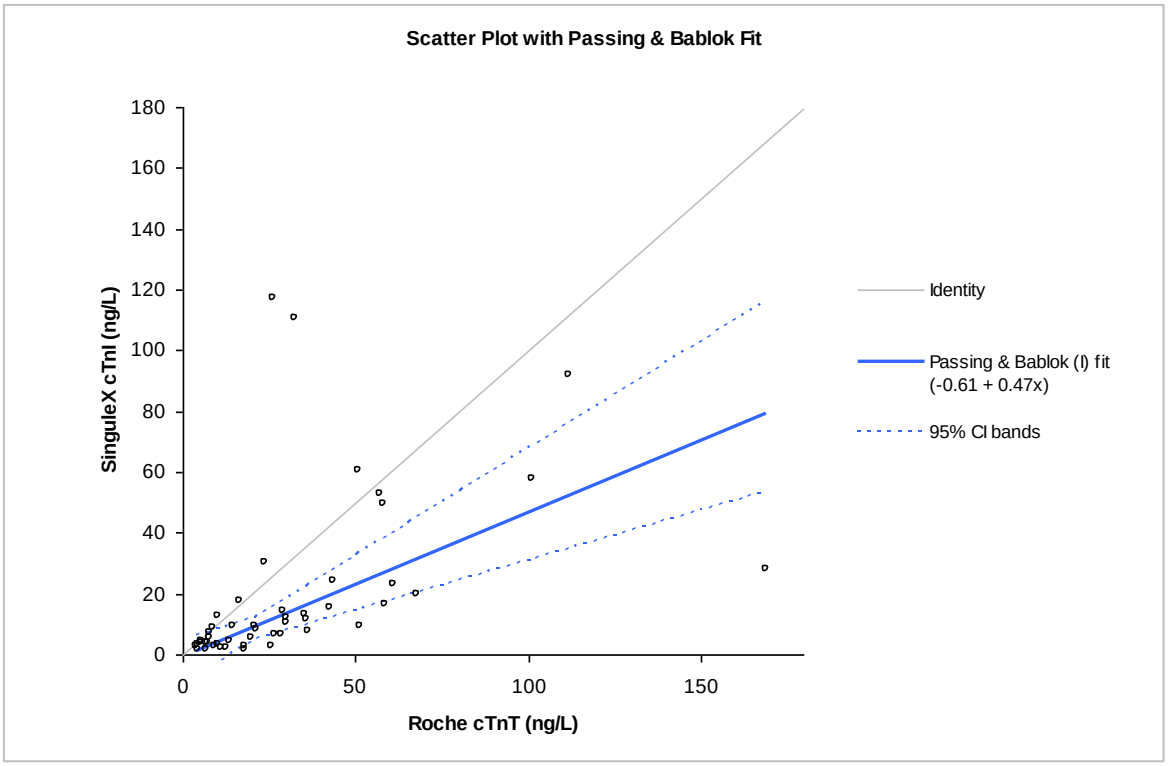


Figure 22. Compilation between the concentrations of the same samples tested on Roche and Singulex.

Figure 23 displays the relation between the different from Singulex concentration to Roche concentration dividing by the mean of all the samples related to the mean of all the samples tested. Therefore, the figure shows the mean percentage of difference between the Singulex and Roche with a result of – 66.8%.

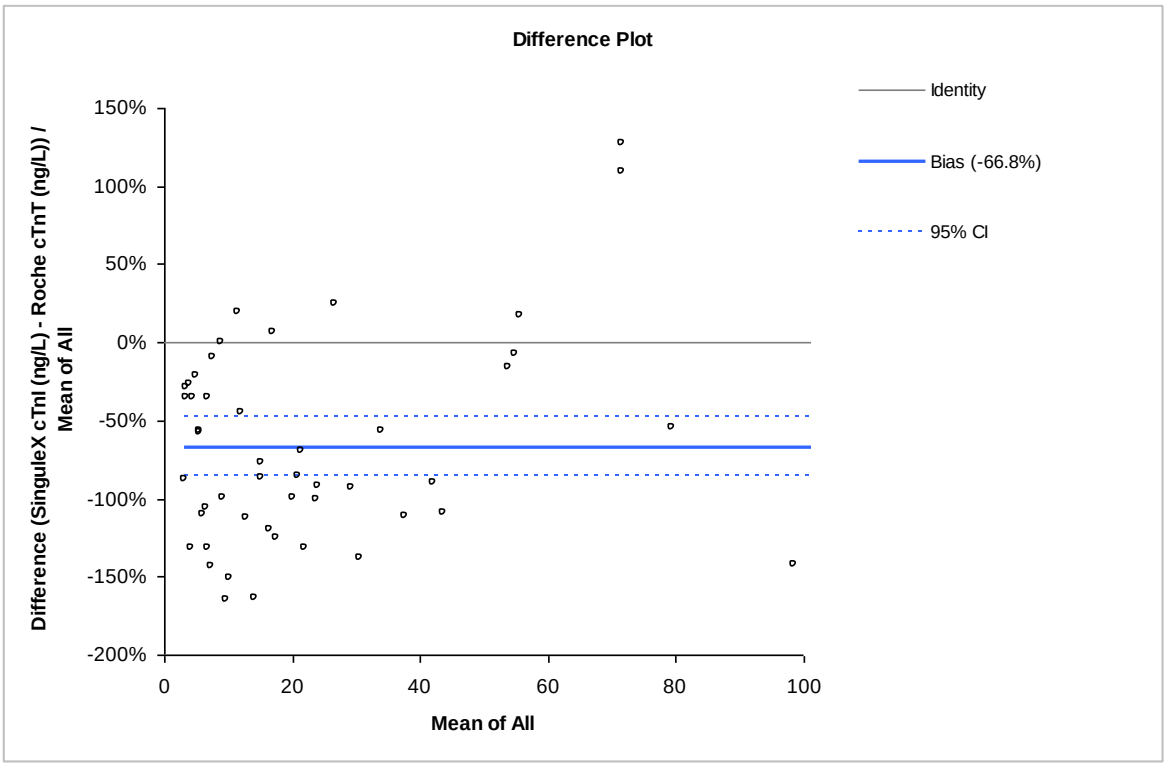


Figure 23. Mean percentage difference between the Roche and the Singulex.

In addition, the mean absolute of the difference between the Singulex and Roche was calculated and shown on Figure 24. The figure shows the relation between the differences from Singulex concentration to Roche concentration related to the mean of all the samples tested. In this figure the result for the mean was -11.8 ng/L.

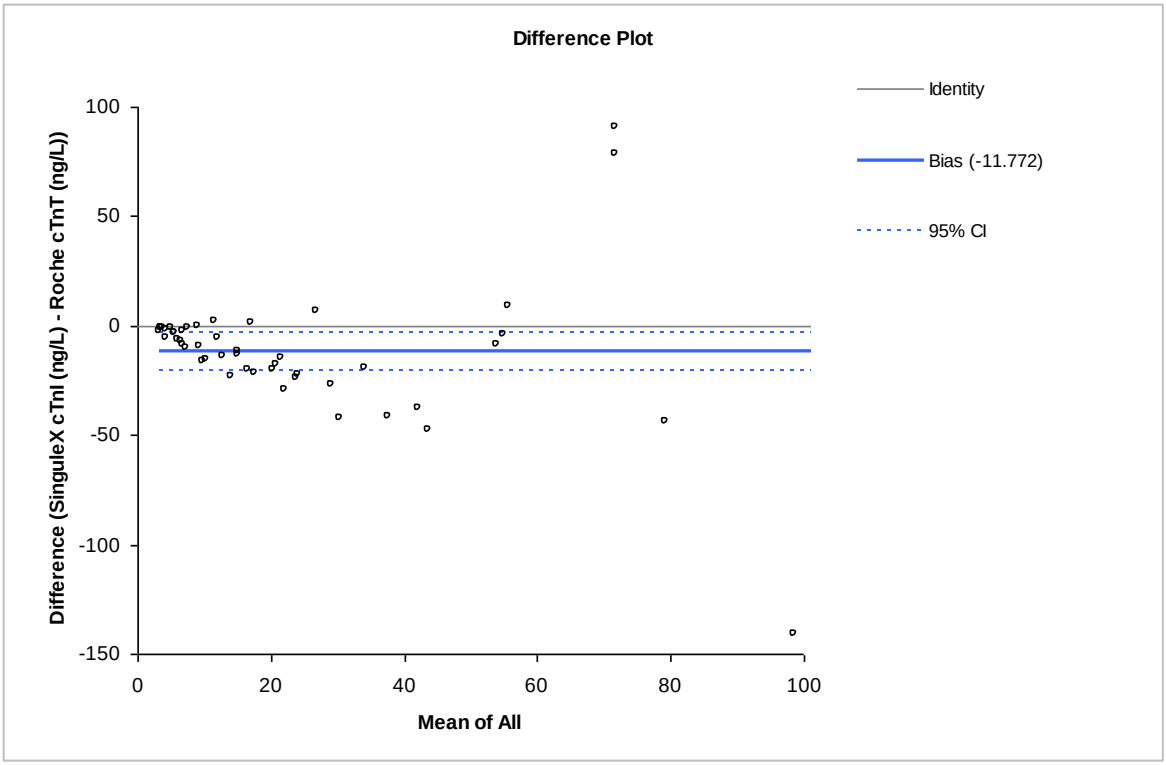


Figure 24. Mean absolute difference between the Roche and the Singulex.

3.4.3. Roche HS cTnT Vs Abbot Architect HS cTnI

From among of all 60 samples tested a total of 47 samples were used in the analysis, 13 samples could not be included because they did not have an absolute value i.e. <2.00 ng/L or <3.00 ng/L in Abbott Architect and Roche, respectively. Samples range from 41.0 to 168.8ng/L for the Roche cTnT method were used to the analysis. Figure 25 shows the compilation between the same samples tested on Roche and Architect.

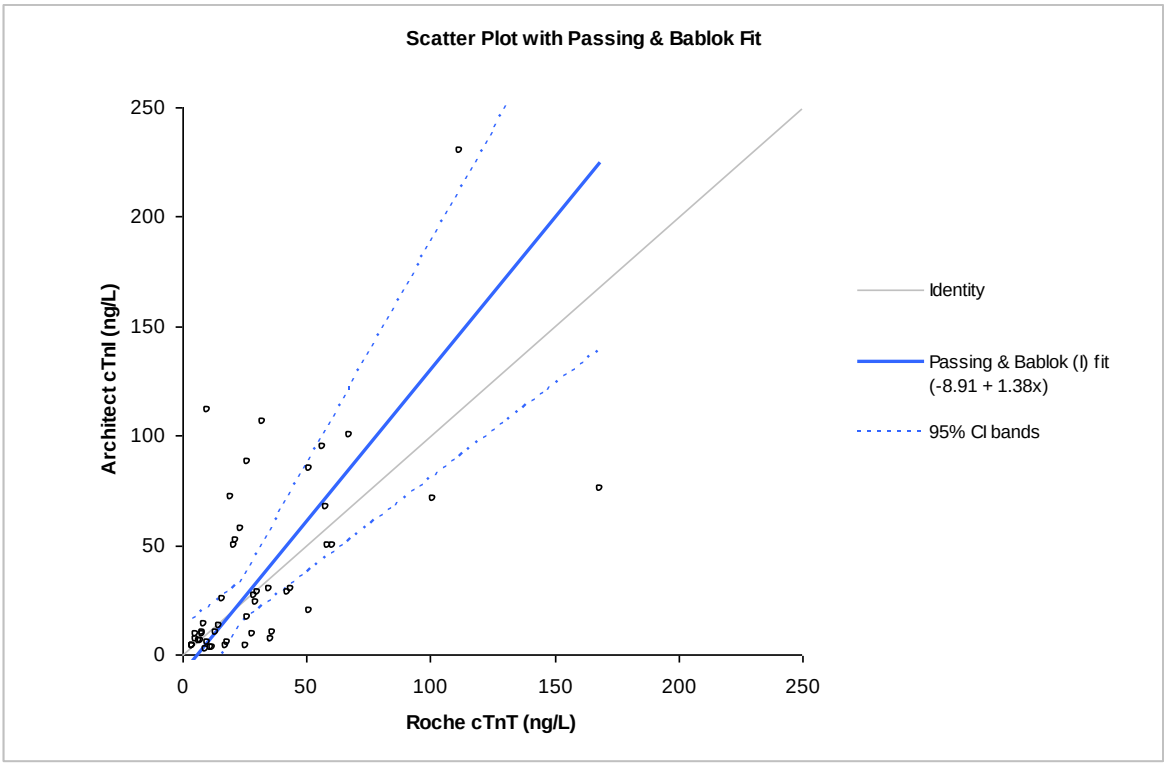


Figure 25. Compilation between the concentrations of the same samples tested on Roche and Architect.

Figure 26 shows the relation between the different from Abbott Architect concentration to Roche concentration dividing by the mean of all the samples related to the mean of all the samples tested. Also, the figure shows the bias of the relation between the Abbott Architect and Rochet with a result of – 10.87%.

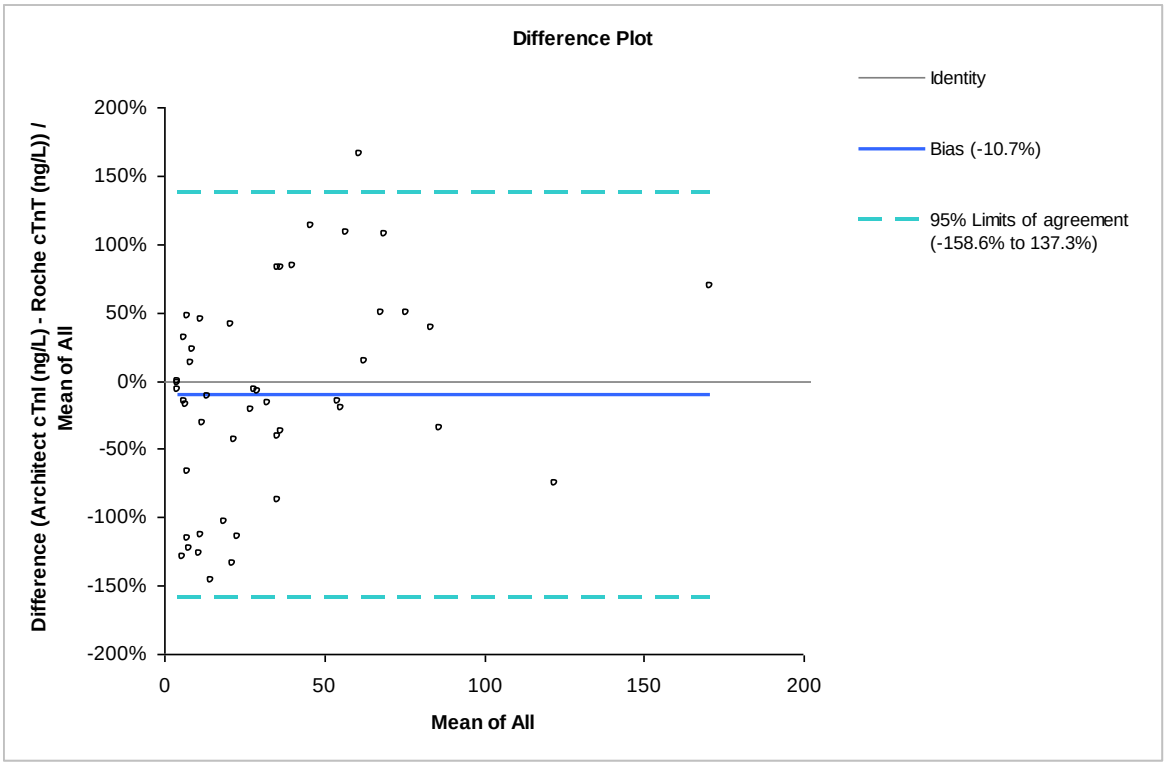


Figure 26. Mean percentage difference between the Roche and the Abbot Architect.

Additionally, the mean absolute of the difference between the Abbott Architect and Roche was calculated and shown on Figure 27. The figure shows the relation between the different from Abbot Architect concentration to Roche concentration related to the mean of all the samples tested. In this figure the result for the mean was 5.3 ng/L.

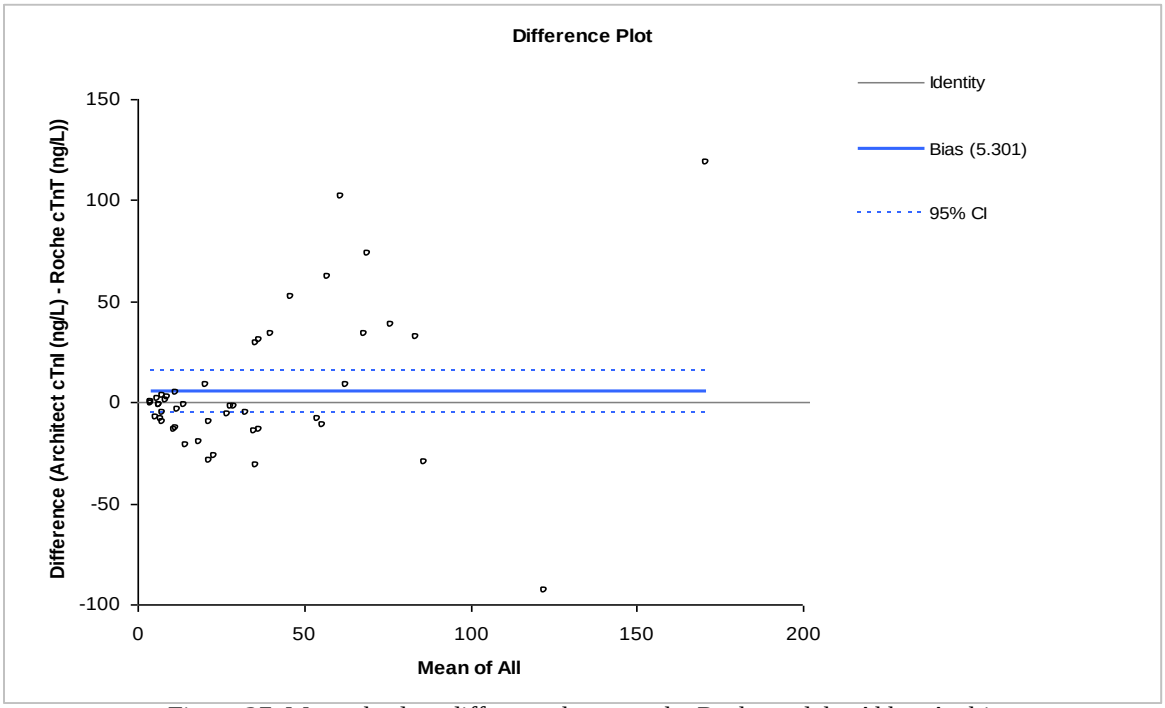


Figure 27. Mean absolute difference between the Roche and the Abbot Architect.

3.5. Stability study

In order to investigate the effects of time and temperature in the stability of Singulex cTnI assay, an experiment was performed. From all aliquots stored at room temperature (+/- 21ºC), -4ºC or -20ºC, two aliquots of each pool were tested on the Sgx Clarity System for 5 subsequent days.

The concentration results for all pools, stored at different temperatures for the five subsequent days are shown on Tables XVI-XX.

Table XVI. Concentration results for the pool no 1 stored at room temperature, 4ºC and -20 ºC for the five subsequent days and the corresponding mean.

	pool no 1, rom temp					**pool no 1, 4ºC**					**pool no 1, -20ºC**				
Day	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
	0.92	0.75	0.60	0.59	0.64	0.92	0.83	0.62	0.65	0.68	0.92	0.94	1.00	0.88	0.87
	0.98	0.73	0.56	0.60	0.51	0.98	0.84	0.72	0.54	0.50	0.98	1.01	0.92	0.97	0.82
Mean	0.95	0.74	0.58	0.60	0.58	0.95	0.84	0.67	0.60	0.59	0.95	0.98	0.96	0.93	0.85

Table XVII. Concentration results for the pool no 2 stored at room temperature, 4ºC and -20 ºC for the five subsequent days and the corresponding mean.

	pool no 2, rom temp					**pool no 2, 4ºC**					**pool no 2, -20ºC**				
Day	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
	1.57	1.22	0.97	1.00	1.13	1.57	1.52	1.22	1.17	1.02	1.57	1.32	1.58	1.33	1.28
	1.59	1.32	1.04	1.04	0.86	1.59	1.34	1.14	1.05	0.99	1.59	1.37	1.47	1.45	1.43
Mean	1.58	1.27	1.01	1.02	1.00	1.58	1.43	1.18	1.11	1.01	1.58	1.35	1.53	1.39	1.36

Table XVIII. Concentration results for the pool no 3 stored at room temperature, 4ºC and -20 ºC for the five subsequent days and the corresponding mean.

	pool no 3, rom temp					**pool no 3, 4ºC**					**pool no 3, -20ºC**				
Day	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
	3.16	2.73	2.36	2.47	1.86	3.16	3.14	2.60	2.82	2.41	3.16	2.23	2.97	3.07	2.86
	2.93	3.00	2.33	2.41	2.05	2.93	3.13	2.60	2.68	2.52	2.93	3.14	3.03	3.21	3.32
Mean	3.05	2.87	2.35	2.44	1.96	3.05	3.14	2.60	2.75	2.47	3.05	3.19	3.00	3.14	3.09

Table XIX. Concentration results for the pool no 4 stored at room temperature, 4ºC and -20 ºC for the five subsequent days and the corresponding mean.

	pool no 4, rom temp					**pool no 4, 4ºC**					**pool no 4, -20ºC**				
Day	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
	83.04	77.62	78.37	69.70	63.08	83.04	72.58	71.51	65.22	71.34	83.04	83.77	83.21	82.32	80.84
	75.83	80.48	73.80	69.05	64.90	75.83	75.79	74.47	68.54	71.50	75.83	84.51	83.71	85.25	77.99
Mean	79.44	79.05	76.09	69.38	63.99	79.44	74.19	72.99	66.88	71.42	79.44	84.14	83.46	83.79	79.42

Table XX. Concentration results for the pool no 5 stored at room temperature, 4ºC and -20 ºC for the five subsequent days and the corresponding mean.

	pool no 5, rom temp					**pool no 5, 4ºC**					**pool no 5, -20ºC**				
Day	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
	314.30	263.51	227.82	227.23	244.37	314.30	282.86	275.82	258.02	257.33	314.30	320.37	320.77	281.68	310.17
	334.92	280.84	242.80	244.98	244.53	334.92	296.14	295.62	268.20	234.07	334.92	322.70	320.51	290.86	301.98
Mean	324.61	272.18	235.31	236.11	244.45	324.61	289.50	285.72	263.11	245.70	324.61	321.54	320.64	286.27	306.18

With all the results and focused on Singulex cTnI stability study some profile figures were made. In this figures (28, 29, 30, 31 and 32) the concentration of each pool, affected by temperature and time, were compared with the subsequent days where the pool were tested.

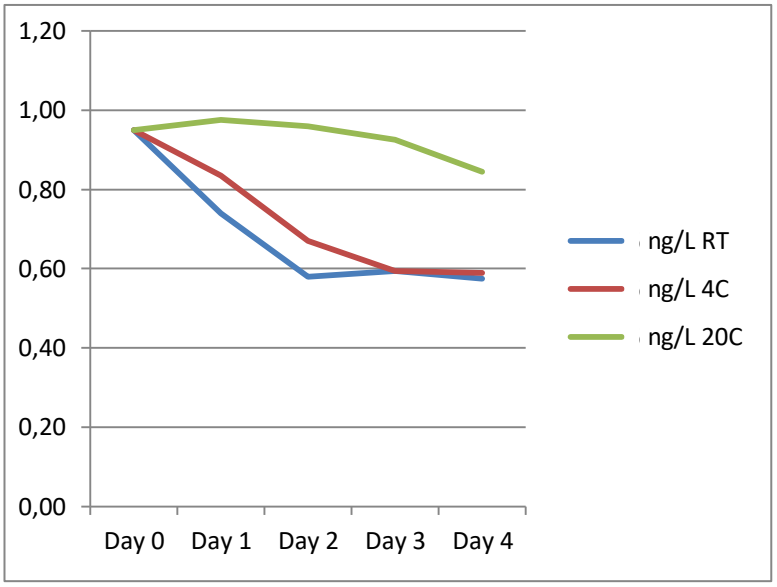


Figure 28. Variation of the concentration of pool no 1, stored at different temperatures, within five days.

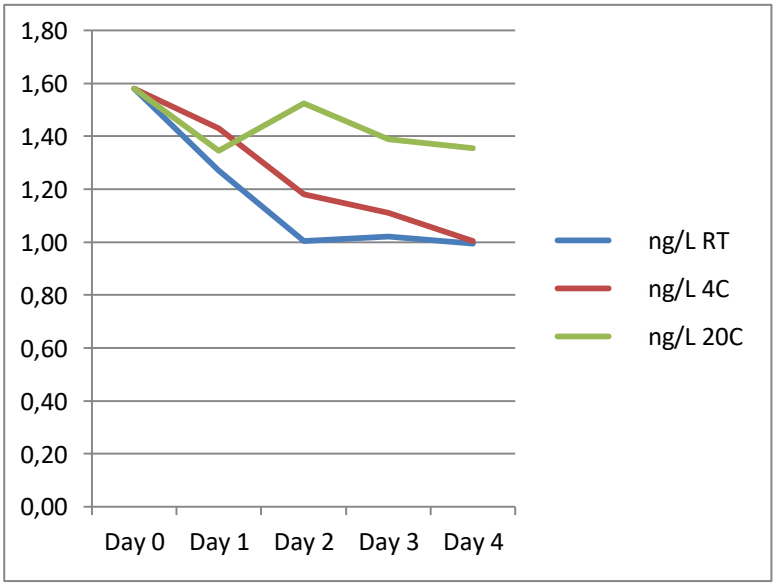


Figure 29. Variation of the concentration of pool no 2, stored at different temperatures, within five days.

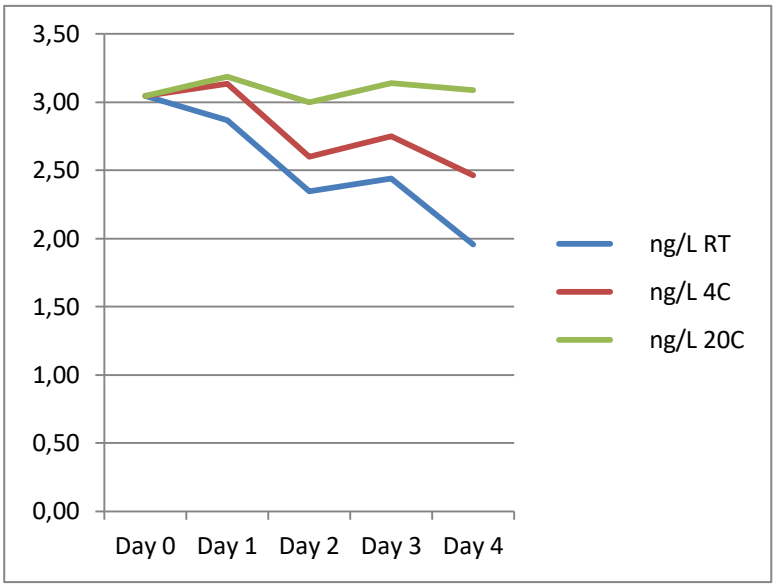


Figure 30. Variation of the concentration of pool no 3, stored at different temperatures, within five days.

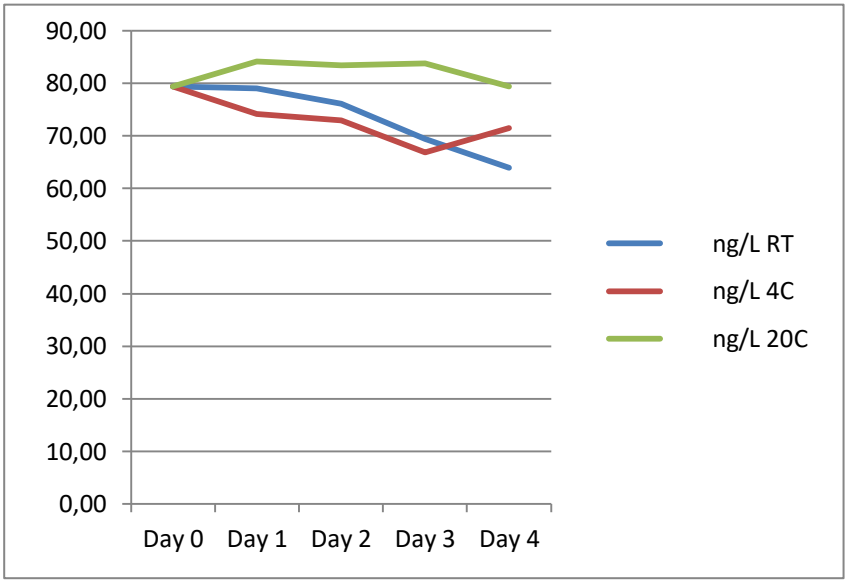


Figure 31. Variation of the concentration of pool no 4, stored at different temperatures, within five days.

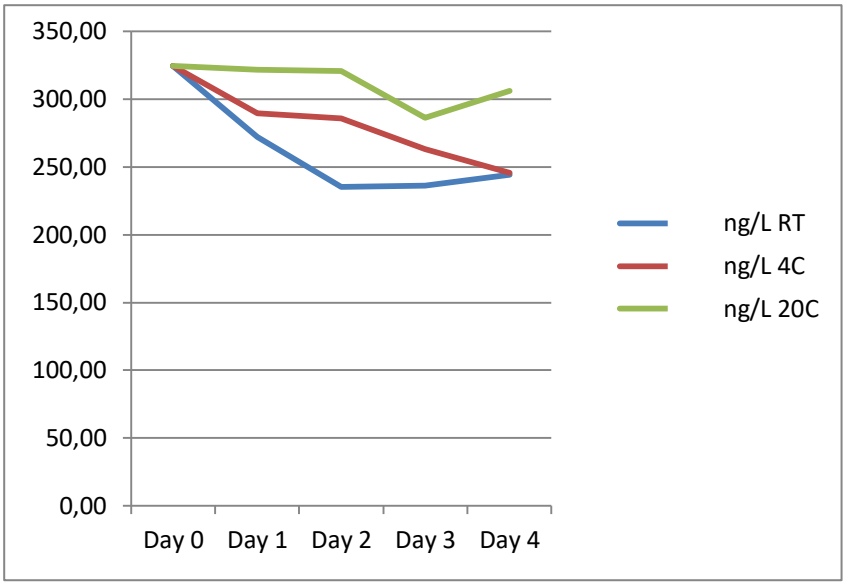


Figure 32. Variation of the concentration of pool no 5, stored at different temperatures, within five days.

Chapter IV –

Discussion

Conclusion and Future Perspectives

4.1 Discussion

Specifications of a perfect cardiac biomarker to diagnose AMI include as a minimum, cardiac specificity, early detection after first symptoms and a long half-life. The presence of an elevation of circulating cTn, the current preferred biomarkers to diagnose AMI, is indicative of myocardial injury. It has been shown that there is a consistently strong direct relationship between increased cTn concentration and the risk of short- and long-term cardiac events and mortality in patients who present with symptoms of ACS in the ED. The main advantage of cTn is its cardiac specificity and its timeframe of release. [15,53,59,60]

High-sensitivity cardiac troponin assay have recently emerged enabling reliable measurement of cTn values in healthy individuals. These assays allow measurement of even low cTn concentrations with high precision, and have been shown to provide high diagnostic accuracy for AMI already at presentation. [47,61]

The ability to rapidly and accurately diagnose patients with symptoms of chest pain to the ED is of critical importance because of the short-term mortality for patients with MI who are mistakenly discharged from the hospital. In addition, diagnostic uncertainty may delay initiation of definitive treatment of patients. Even for the physician within the ED who wants a precise assay at the 99th percentile cutoff, there is an interest that undetectable and/or low hs-cTn result may identify patients at low risk of ACS and possibly suitable for early discharge.[30,58,62]

With the development of more sensitive and precise hs-cTn assays, and their ability to determine small differences in cTn over time, new possibilities become available to improve risk stratification and to facilitate the identification of patients at risk for a cardiovascular event. The present study undertook an analytical validation of a novel high sensitivity assay to determine if the performance characteristics and laboratory usability would allow it to perform in such a role.

4.1.1. Percent normal study

In the present study hs cTnI was measured in a large, presumed, healthy population. Our results demonstrated that the Singulex hs-cTnI assay measured 100% of concentrations in all healthy subjects, resulting in a concentration range of 0.13-11.27

ng/L. Most current cTn assays do not detect cTn in 50% of apparently healthy individuals whereas high-sensitivity assays do and with some, detection may be as high as in 90%. [55] However, although the population studied was assumed as healthy, it is known that increased hs-cTnI or cTnT concentrations can result from other pathological etiologies that cause myocardial cell injury, cell death, and release of cTn into the blood. It is therefore possible that some of the population studied had subclinical disease. [63]

According with the literature, our observations also demonstrated a significant difference between men and women. Previous studies reported substantially higher 99th percentile values in males *vs* females, using four hs-cTnI assays (Abbott ARCHITECT hs-cTnI; Beckan hs-cTnI; Siemens Dimension Vista hs-cTnI; Singulex Erenna hs-cTnI). Moreover, previous studies have shown a sex difference for the hs-cTnT assay. [15,44,64]

Ischemic heart disease is more common in males, but the population studied was presumed to be healthy. So the explanation may be that as male hearts are on average larger than female hearts, a sex difference in distribution of troponin values would be expected. [15]

The observations in the present study also demonstrated an association between the values for cTnI and age of the healthy individuals, with values tending to increase with age. The project based on the Biomarkers for Cardiovascular Risk Assessment in Europe (BiomarCaRe) had suggesting that the addition of troponin I led to a greater incremental risk prediction with rising age. [64,65]

The troponin I concentrations in apparently health subjects are continuously associated with fatal cardiovascular events and to a lesser extent with incident cardiovascular disease as well as overall mortality. Risk phenotypes such as the degree of atherosclerosis, ventricular hypertrophy and vascular stiffness together with very low circulating levels of troponin I explain a substantial proportional of cardiovascular risk. However, elevated concentration at baseline is most probably due to subclinical cardiac pathology which increases the risk of cardiovascular death. Higher troponin concentrations may reflect subclinical CAD and identify those at greatest risk who could benefit from targeted preventative therapies. [64,65]

4.1.2. Precision study

For the clinical utilization of a high sensitivity assay, very short time intervals between sequential sampling was recommended, with samples taken on admission, 90 min, 2h and 3h post admission and possibly admission and 1h from admission. In order to use such shorter time interval between high-sensitivity cardiac troponin measurements safely a high degree of repeatability is required. [46,66]

Our results from the pools tested on Sgx Clarity System have shown a CV of <10% for all the pools, as well as the same pools tested on Cobas 8000, for cTnT. The results have shown a lower CV in the high concentrations pools and demonstrate very high precision at higher levels of cTnI. On the other hand, the results for the intermediate precision pools using Roche cTnT assay are consistent with a previous study using the Abbot ARCHITECT hs-cTnI assay, where the hs-cTnI assay has superior precision at lower concentrations.[62]

When compared, the results on Sgx Clarity System for hs-cTnI assay and on Cobas 8000 for hs-cTnT assay the concentrations values were clearly different. In the same pools, the concentration of cTnI was larger when compared with cTnT mainly because they are different analytes and each have their own biological kinetic characteristics.[67] However, consistently with the literature, it is well-known that both troponins I and T levels in patients without ACS are at least part linked to renal function. The fact of weight of troponin T (37kDa) is bigger than troponin I (22.5kDa) could be an explanation. [37,67]

For the repeatability precision study, as for the intermediate precision study, all the CV represented a value below 10%, representing a high precision and repeatability of the assay. However, because we are using a high sensitivity assay it is important to identify a superior precision at low concentrations values, as well as identified at high concentrations values.

With this precision study we concluded that Sgx Clarity System is able to processes the same, and different samples, in different times with a high precision. High precision and repeatability is critical for the clinical use of the assay, especially when we need to compare small changes in concentration of cTn over short time intervals, in order to discharge or admit the patient and define the correct treatment for them.

4.1.3. Linearity study

In order to test the linearity of the assay, and verify the capacity of the instrument of give a direct relation between the results and the concentration of the analyte, in this case cTnI, a linearity study was performed. The assay showed excellent linearity, which allows us to conclude that measured changes in value across the assay range will be reliable.

4.1.4. Patient comparison study

Nowadays, comparability testing for hs-cTnI across hospital and laboratories is essential because within a city there may be downtime for the analyzers occurring as a result of system failure and samples may need to be sent to another site for testing or more commonly there will be patients transferred from one hospital to another. A previous study indicated that agreement, as assessed by CV, is superior for hs-cTnI as compared to cTnI.[62]

Each cTnI assay uses different antibodies, calibrators, control materials, and detection techniques, limiting laboratory and multicenter study comparison. [15] In the present study we described and quantified between and within-method differences between cTnI assays and between cTnI and cTnT assays.

Overall, troponin was detectable by the Abbot Architect hs-cTnI assay in 51/60 samples (85%), by Roche hs-cTnT assay in 49/60 samples (81,7%) and by Singulex hs-cTnI assay in 60 samples (100%). As mentioned before, in this study Sgx Clarity System has detected cTnI concentration in all the samples tested.

A difference between the results was expected due to the analytical features mentioned above as well as factors presented in patient serum, such as anti-cardiac troponin antibodies, hemolysis, icterus, lipemia and drugs which may affect individual assays differently. [33]

4.1.5. Stability study

In this study all the variables were constant for all samples and all measurements on the analyzer were made under similar conditions. Pools with different concentration were

stored at different temperatures and tested on the Sgx Clarity System for 5 subsequent days.

The results have shown a time and temperature-dependent degradation in all the different serum pools.

It has to be noticed that stability of cTnI in the pools stored at room temperature was lower as compared to those stored at -20ºC, independent of the pools concentration.

All the different concentrated pools have shown a decreased in cTnI concentration in the pools stored at room temperature, across the 5 subsequent days, when compared with those stored at -20ºC, event that there is a decreased of cTnI concentration, the difference was not so clear. Another fact is the discrepancy seen in the samples with lower analyte concentrations. In the first 3 pools (lowest concentrated pools), the results showed a very marked decrease. However in the last two higher concentrated pools the difference was not so apparent, which means that samples with higher concentration are more stable when stored at -20ºC or even stored at room temperature.

In terms of clinical value, this means that high concentrations can be measured after sample storage. Hence retrospective confirmation of significant troponin elevation can be obtained. However, low concentrations may not be reliably measured if the sample has not been stored frozen at -20°.

4.2 Conclusion and Future Perspectives

The appropriate use of hs-assays will improve clinical care. In clinical practice hs-assays will allow the early detection of small cTn changes evolving from low cTn concentrations within the reference interval to small increases above the 99th percentile value. This will be important in improving the understanding of the role of absolute concentration changes and it will improve clinical specificity for MI compared to another myocardial injury mechanism.

Currently, some assays already proposed a very rapid diagnosis. However they need a high precision at low levels. Even in the chronic disease detected there is a need of high sensitivity to monitor them. Sgx Clarity System is a potential analyzer in an analytical performance view. However it doesn't provide yet the usability necessary for a routine laboratory.

Sgx Clarity System is a batch analyser with capacity for 48 samples per run, however in a clinical laboratory where we constantly receive samples we should be able to load samples while the others are running. Also, the analytical time is too long and for a routine laboratory with a big amount of workload where the turnaround time should be as shorter as possible a quick assay is required. Another disadvantage of this instrument is the set up time that it is also too long, as well as the calibrators and controls processing time. With all this details we concluded that however the instrument is perfectly satisfactory in an analytical performance and it is sensitive, measuring small concentrations of cTnI, it is not ready to be in a routine laboratory.

The prospective evaluation of this assay has yet been fully characterized in a larger population of patients presenting to the ED with chest pain. As a future research defining the diagnostic accuracy it will be interesting to perform a study that includes a delta change. A follow-up study is currently underway in our laboratory to examine specimens from patients with a diagnosis of MI.

We note, as a potential limitation, the possibility that with such sensitive limits of detection of the assay, low-level nonspecific binding events might contribute to cTnI measurements. Also, the presumably healthy study participants used in the percent normal study were identified via health conditions and not by clinical using additional screening

tools such as imaging, ECG or measurement of a natriuretic peptide, as a surrogate myocardial dysfunction biomarker.

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